Medium-Engineering für enzymatische Reaktionen am Beispiel von Hydroxynitril Lyasen und Alkoholdehydrogenasen

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für meine Familie

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Kurzfassung

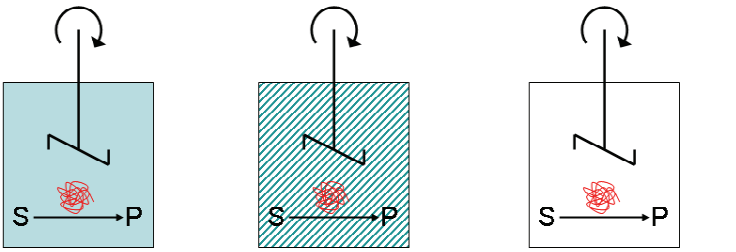
Medium-Engineering für enzymatische Reaktionen am Beispiel von Hydroxynitril Lyasen und Alkoholdehydrogenasen

In den letzten Jahren sind Biokatalysatoren ein unverzichtbarer Bestandteil für zahlreiche industrielle Prozesse geworden. Die ausgesprochen hohen Stereo-, Regio- und Enantioselektivitäten gepaart mit deutlich geringeren Neben- und Abfallprodukten stellen wichtige Vorteile gegenüber den klassischen chemischen Verfahren dar.

Bedingt durch die Verwendung nicht-natürlicher Substrate werden oftmals Cosolventien hinzugesetzt um beispielsweise die Löslichkeiten der Reaktanden zu erhöhen. Diese gezielte Beeinflussung der Reaktionsparameter, auch ‚*Medium Engineering*' genannt, ermöglicht die Manipulation der kinetischen und thermodynamischen Parameter hin zu höheren Produktivitäten und Selektivitäten.

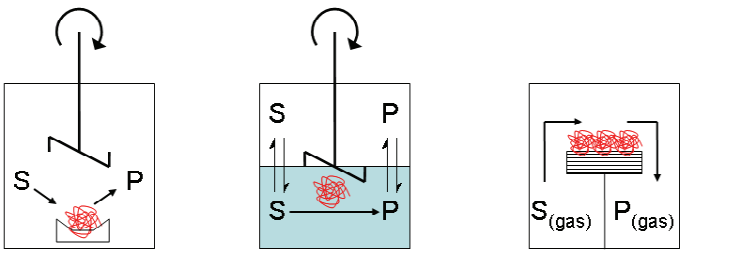
In der vorliegenden Arbeit wurden am Beispiel von Hydroxynitril Lyase- und Alkoholdehydrogenase-katalysierten Reaktionen verschiedene Reaktionskonzepte mit organischen Lösungsmitteln hinsichtlich ihrer Praktikabilität zur Überwindung von verschiedensten Limitierungen untersucht.

<u>Einphasensysteme</u>



pur wäßriges system	wäßriges System mit wassermischbaren Cosolventien	organisches Lösungsmittel mit gelöstem Enzym

<u>Mehrphasensysteme</u>



organisches Lösungsmittel mit suspendiertem oder immobilisiertem Enzym	wäßrig-organisches Zweiphasensystem	Gasphasenreaktion

Nach Abschluss der Bewertungen konnten mehrfach Produkte im Gramm-Maßstab gewonnen werden, beispielsweise mittels der Hydroxynitril Lyase aus *Manihot esculenta* 5 x 50 g enantiomerenreines Produkt (Mandelsäurederivate).

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Publikation 1
"Influence of water-miscible organic solvents on kinetics and enantioselectivity of the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis*"
Schumacher, J.; Eckstein, M.; Kragl, U., *Biotechnology Journal*, 1, 574-581 (2006)

Publikation 2
"Hydroxynitrile lyase in organic solvent free systems to overcome thermodynamic limitations"
von Langermann, J.; Mell, A.; Paetzold, E.; Daußmann, T.; Kragl, U., *Advanced Synthesis and Catalysis*, 349, 1418-1424 (2007)

Publikation 3
"A new (*R*)-selective Hydroxynitrile Lyase from *Arabidopsis thaliana* with an alpha/beta-Hydrolase fold"
Andexer, J.; **von Langermann, J.**; Mell, A.; Bocola, M.; Kragl, U.; Eggert, T.; Pohl, M., *Angewandte Chemie - International Edition*, 46, 8679-8681 (2007)

Publikation 4
"Hydroxynitrile lyase catalyzed cyanohydrin synthesis at high pH-values"
von Langermann, J.; Guterl, J.-K.; Pohl, M.; Wajant, H.; Kragl, U., *Bioprocess and Biosystems Engineering*, 133, 155-161 (2008)

Publikation 5
"Enzyme catalysis in non-aqueous media – past-present-future"
Dreyer, S.; Lembrecht, J.; **Schumacher, J.**, Kragl, U. in R. Patel (Editor) "*Biocatalysis in the Pharmaceutical and Biotechnology Industries*" (2006), CRC-Press, Taylor & Francis Group, Boca Raton, pp 791-828

Publikation 6
"Hydroxynitrile lyase catalysed synthesis of enantiopure (*S*)-acetophenone cyanohydrins"
von Langermann, J.; Mell, A.; Paetzold, E.; Kragl, U. in J. Whittal (Editor) "*Practical Methods in Biocatalysis and Biotransformations*", John Wiley & Sons Ltd, accepted

9 Anhang

Symbol- und Abkürzungsverzeichnis

7-ACA	7-Aminocefalosporansäure
a_{H2O}	Wasseraktivität (auch a_W)
ADH	Alkoholdehdrogenase
ADH-'A'	Alkoholdehydrogenase aus *Rhodococcus ruber*
ADH-LB	Alkoholdehydrogenase aus *Lactobacillus brevis*
Asp	Asparagin
a_W	Wasseraktivität
b	Effektorkonstante
C1	Chemische Einheit mit einem Kohlenstoffatom
DMSO	Dimethylsulfoxid
E.C.	**E**nzyme **C**ommission
e.e.	Enantiomerenüberschuss
ESS	Doppel-Substrat-Komplex
E_T30	Dimroth-Reichardt-Parameter
f	Fugazität
[H]	Hydrierungsreaktion (allgemein)
*Hb*HNL	Hydroxynitril Lyase aus *Hevea brasiliensis*
HCN	Blausäure
His	Histidin
HIV	Humanes Immundefizienz-Virus
HL-ADH	Pferdeleber-Alkoholdehydrogenase
HNL	Hydroxynitril Lyase
IUBMB	**I**nternational **U**nion of **B**iochemistry and **M**olecular **B**iology
Kat*	Aktiver Katalysator
kDa	1000 Da (1 Da = 1/12 der Masse des Kohlenstoff-Isotops ^{12}C, nicht SI-konforme Einheit)
LB-ADH	Alkoholdehydrogenase aus *Lactobacillus brevis*
LM	Lösungsmittel
logP	Verteilungskoeffizient im System Wasser/*n*-Octanol
*Lu*HNL	Hydroxynitril Lyase aus *Linum usitattissimum*
Me	Methyl
*Me*HNL	Hydroxynitril Lyase aus *Manihot esculenta*
mmol/L	Millimol je Liter

NMP	N-Methyl-2-pyrrolidon
NAD(P)H	Reduzierte Form des Nicotinamid-adenin-dinucleotid-(phosphat)
$NAD(P)^+$	Nicotinamid-adenin-dinucleotid-(phosphat)
p	Partialdruck
P	Produkt
*Pa*HNL	Hydroxynitril Lyase aus *Prunus amygdalus*
S	Substrat
Ser	Serin
*Sb*HNL	Hydroxynitril Lyase aus *Sorghum bicolor*
TB-ADH	Alkoholdehydrogenase aus *Thermoanaerobacter brockii*
Thr	Threonin
TMSCl	Trimethylchlorsilan
u.a.	Unter anderem
Vgl.	vergleiche
Vol%	Volumenprozent (auch % (v/v))
Y-ADH	Alkoholdehydrogenase aus Hefe
ZnCPC	Zinksalz des Cephalosporin C

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1 Einleitung

Biotransformationen zur großtechnischen Gewinnung von chemischen Substanzen stellen seit einigen Jahren eine ernstzunehmende Alternative gegenüber den klassischen synthetischen Verfahren dar **[Festel *et al.* 2004, Liese *et al.* 2006, Schoemaker *et al.* 2003, Wandrey *et al.* 2000]**. Die Hauptvorteile der Biokatalysatoren liegen in ihren hohen Stereo-, Regio- und Enantioselektivitäten, wobei meist weniger Neben- und sonstige Abfallprodukte auftreten **[Faber 2004]**. Zusätzlich sind die Reaktionsbedingungen in vielen Fällen sehr moderat und erfordern deutlich geringere Anforderungen an die Prozesstechnik, da beispielsweise weniger aufwendige Reaktionsbedingungen (z. Bsp. wässrige Reaktionsmedien) verwendet werden können. Dies führt zu geringeren Sicherheitsbestimmungen und Einsparungen in den Produktionskosten **[Wohlgemuth 2006]**.

Enzyme werden nach der Einteilung der IUBMB (**I**nternational **U**nion of **B**iochemistry and **M**olecular **B**iology) von 1961 in 6 Klassen gegliedert (Tabelle 1-1) **[Silverman 2002]**.

Tabelle 1-1: IUBMB-Einteilung der Enzyme

Enzymgruppe	Katalysierte Reaktion	Enzym-Beispiel
E.C. 1 – Oxidoreduktasen	Redoxreaktionen	Alkoholdehydrogenase
E.C. 2 – Transferasen	Transferreaktionen von einzelnen funktionellen Gruppen	ω-Transaminase
E.C. 3 – Hydrolasen	reversible hydrolytische Spaltungsreaktionen	Lipase
E.C. 4 – Lyasen	Bindungsspaltung und –aufbau	Hydroxynitril Lyase
E.C. 5 – Isomerasen	Isomerisierungs- und Racemisierungsreaktionen	Glucoseisomerase
E.C. 6 – Ligasen	Bindungsaufbau unter Energieverbrauch (z. Bsp. ATP)	Carboxylase

Zur Zeit sind mehr als 10000 natürliche Enzyme bekannt, wobei nur 3000 davon charakterisiert wurden. Nur ein sehr kleiner Teil davon (cirka 150 Enzyme findet als als reines Protein oder Rohextrakt Verwendung in der großtechnischen Synthese **[Kutter 2007]**. Zusätzlich ermöglicht der Einsatz der modernen Molekularbiologie die Darstellung vieler weiterer Enzymvarianten, so dass auch das jeweilige Substratspektrum modifiziert werden kann **[Drepper *et al.* 2006]**.

Es sind aktuell zahlreiche biotechnologische Produktionsprozesse für bulk-, Agrar- und Feinchemikalien im multi-Tonnen-Maßstab verfügbar (Tabelle 1-2) **[Aleu *et al.* 2006, Bott *et al.* 2004, Liese *et al.* 2006, Wandrey *et al.* 2000]**. Die Bezeichnung hierfür lautet: weiße Biotechnologie **[Fessner 2005]**. Eine wichtige Bedeutung hat der großtechnische Einsatz von Hefe, welche durch die großen Produktmengen innerhalb der Ethanolproduktion eine herausragende Stellung erlangt hat **[Hermann *et al.* 2007]**.

Tabelle 1-2: Industrielle biotechnologische Produktionsprozesse

Produktion t/a	Produkt	Biokatalysator	Hersteller
>18.500.000	(Bio-)Ethanol	Hefe (mikrobielle Fermentation)	verschiedene
8.000.000	Fruktose-Glukose-Sirup (engl.: HFCS)	Glucoseisomerase	verschiedene
1.500.000	L-Glutamat	z.Bsp.: *Brevibacterium favum* (Fermentation)	Ajinomoto
1.200.000	(Bio-)1-Butanol	z.Bsp.: *Clostridium aceto-butylicum* (Fermentation)	verschiedene
1.000.000	Zitronensäure	transgene Variante von *Aspergillus niger*	verschiedene
700.000	L-Lysin	*Corynebacterium glutamicum*	Evonik, Ajinomoto, BASF
190.000	Essigsäure	*Acetobacter* oder *Gluconobacter*-Arten	verschiedene
100.000	Acrylamid	Nitrilhydratase	Mitsubishi Rayon
50.000	L-Sorbose	*Gluconobacter suboxidans*	verschiedene
30.000	Riboflavin (Vit. B_2)	Ganze Zellen bzw. Pilze	DSM, BASF
7.000	7-ACA	D-Aminosäure Oxidase, Glutaryl Amidase	Novartis Pharma AG
> 1	Sekundäre Alkohole	Alkoholdehydrogenase	Wacker **[Daussmann 2005]**
> 1	(*S*)-2-Chlor-Mandelsäurenitril	Hydroxynitril Lyase	Archimica **[Wisdom 2007]**

Im Jahr 2004 betrug der Anteil der biotechnologisch hergestellten chemischen Produkte nur 5% (30 Mrd. US-$) vom Gesamtumsatz der chemischen Industrie, aber für die kommenden Jahre wurde ein deutlicher Anstieg bis auf 20 % (310 Mrd. US-$) prognostiziert **[Bott *et al.* 2004, Festel *et al.* 2004]** (Abbildung 1-1). Hervorzuheben ist an dieser Stelle der Bereich der Feinchemikalien, welcher 2010 ca. 60 % des

Umsatzvolumens ausmachen wird. Dies beinhaltet neben den klassischen Produkten Insulin, Wachstumshormone und Gentherapeutika auch chirale Moleküle für die Agrar- und Pharmaindustrie. Des Weiteren wird der Bereich der Nutzung nachwachsender Rohstoffe in Zukunft deutlich durch biotechnologische Verfahren bestimmt werden. Für biotechnologische Verfahren mit dem Schwerpunkt der Gewinnung organischer Substanzen direkt durch Fermentation wird in Zukunft einen Marktanteil von bis zu 15 % erwartet **[Festel *et al.* 2004, Hatti-Kaul *et al.* 2007]**.

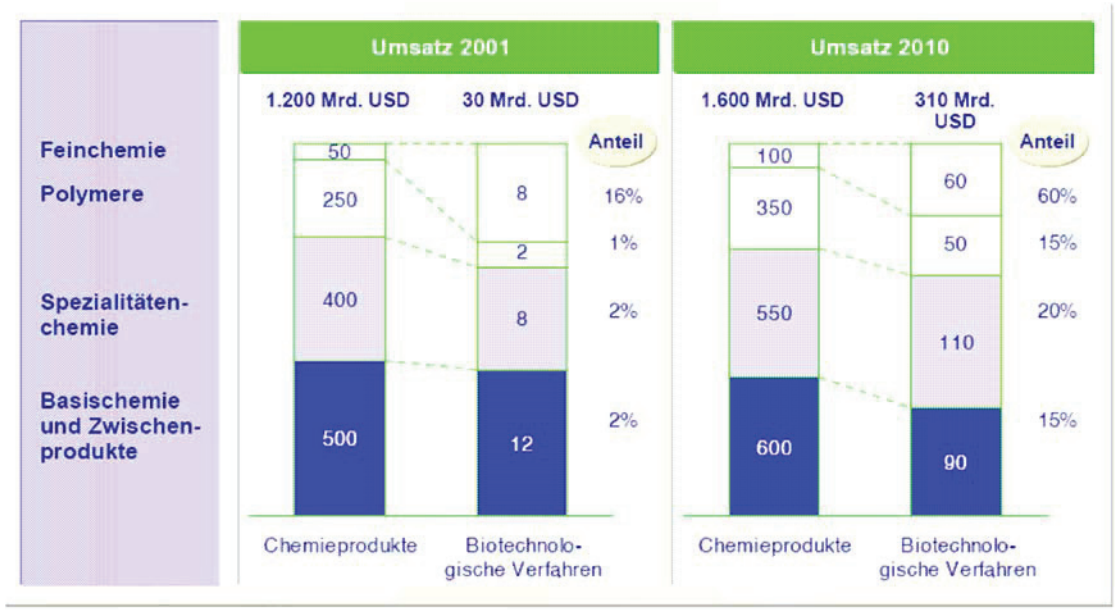


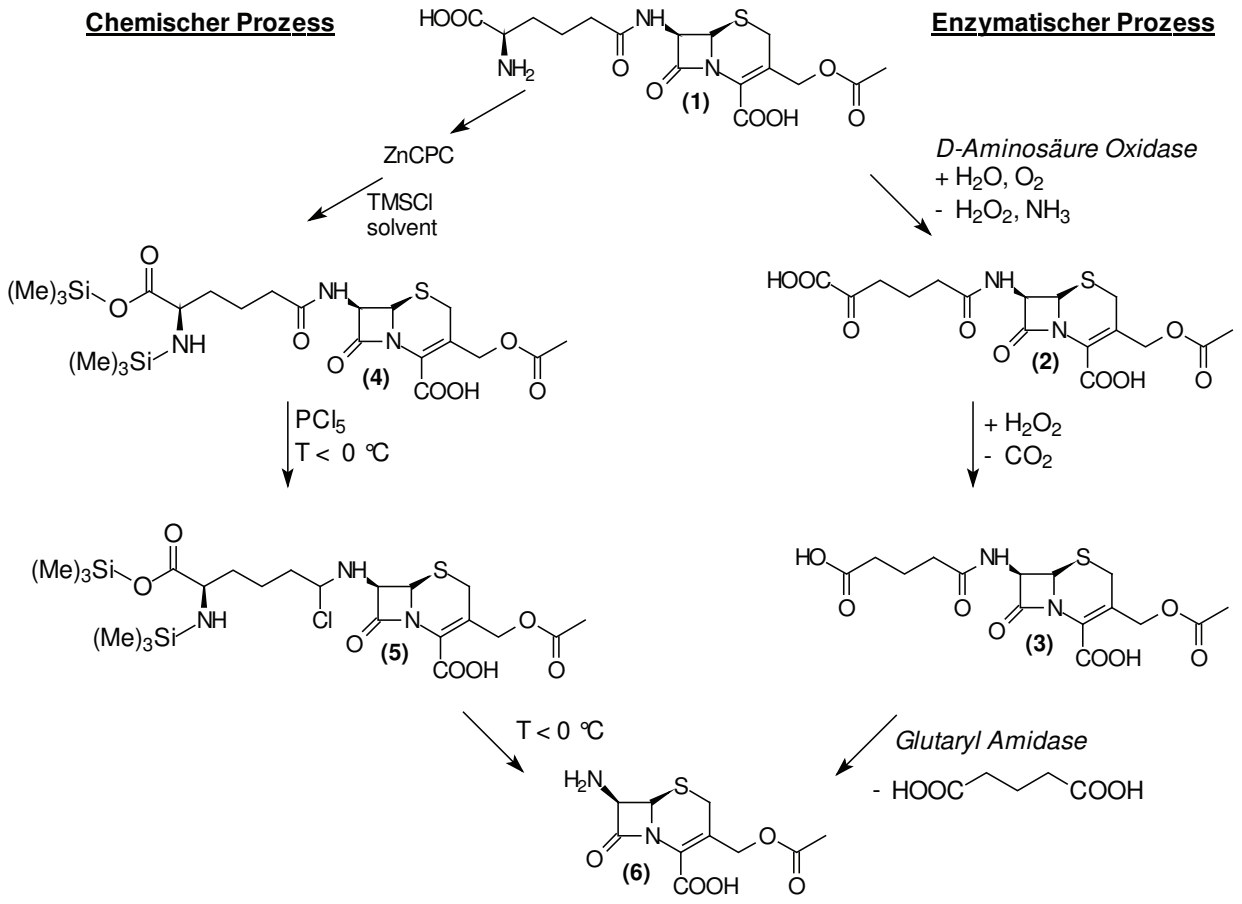
Abbildung 1-1: Anstieg des Verkaufumsatzes aus biotechnologischen Produktionsverfahren bis 2010

Tabelle 1-3: Prozesskonditionen in der 7-ACA-Synthese

Prozesskonditionen	Chemische Route	biokatalytische Route
Temperatur	0 °C	25 – 30 °C
pH-Wert	(organisches LM)	7.3 – 8.3
Lösungsmittel	CH_2Cl_2	wässrig
Abgase	7.5 kg[1]	1.0 kg[1]
Zinkabfälle	1.8 t[1]	0 t[1]
Mutterlaugen (thermische Entsorgung)	29 t[1]	0.3 t[1]
Zusatz-Kosten für den Umweltschutz (bezogen auf das Produkt)	100 %	10 %

[1] bezogen auf 1 t 7-ACA

Zum Teil haben schon heute biokatalytische Reaktionspfade bestehende klassische Verfahren verdrängt **[Wandrey *et al.* 2000]**. Beispielsweise wurde die klassische Zn-katalysierte Synthese von 7-Aminocefalosporansäure (7-ACA) durch eine zweistufige biokatalytische Alternativroute ersetzt, was mit einer großen Kosten- und Abfallreduktion einherging **[Liese *et al.* 2006]** (Tabelle 1-3 und Abbildung 1-2).



(1) Cephalosporin C

(2) a-Ketoadipinyl-7-Amino-Cephalosporinsäure

(3) Glutaryl-7-Amino-Cephalosporinsäure

(4) trimethylsilyliertes Produkt

(5) Imid Chlorid

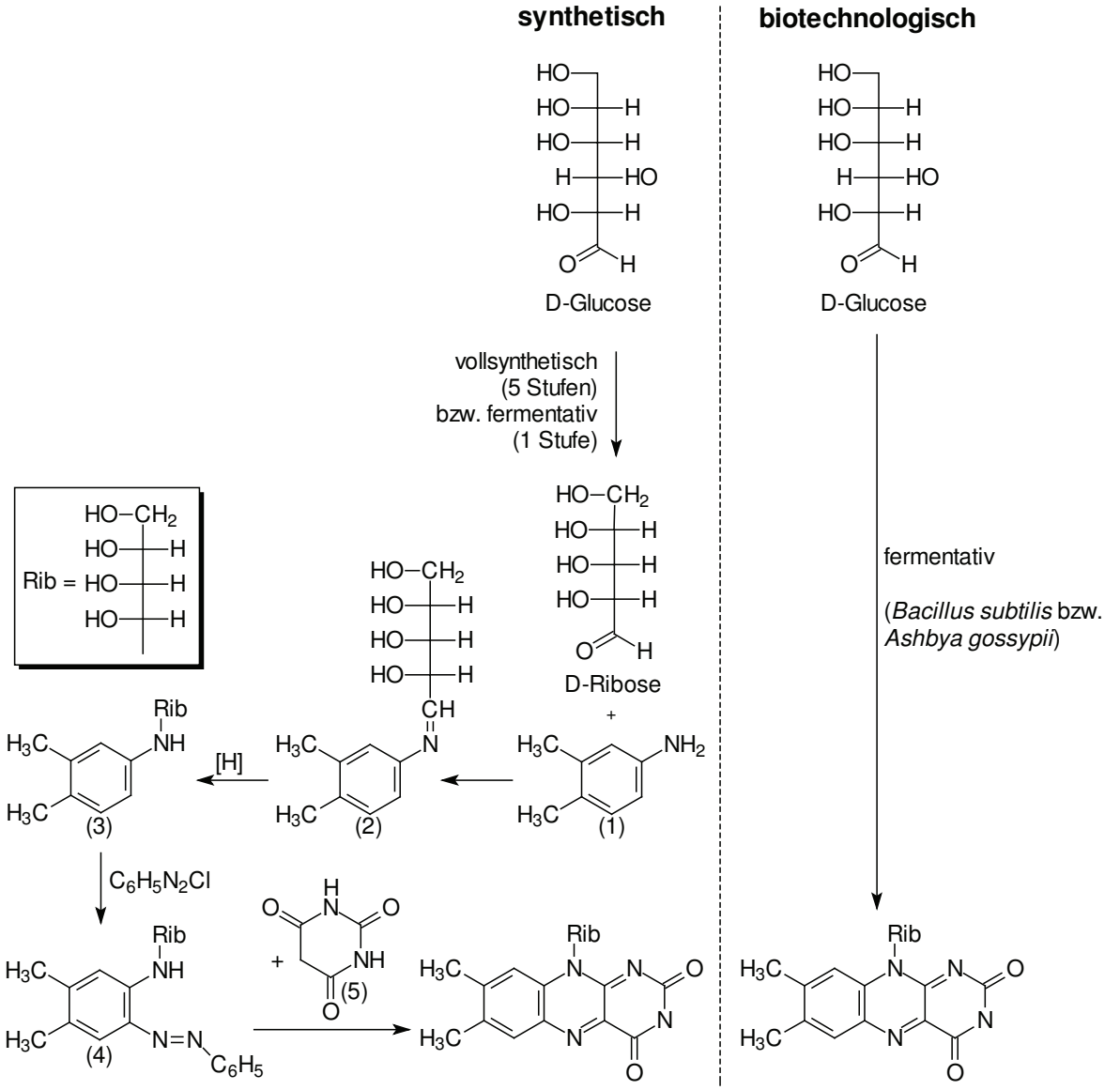
(6) 7-Amino-Cephalosporinsäure

ZnCPC – Zinksalz des Cephalosporin C

TMSCl - Trimethylchlorsilan

Abbildung 1-2: 7-ACA-Synthese

Die Gegenüberstellung der klassischen und der biotechnologischen Syntheseroute zeigt deutlich den großen Vorteil des biokatalytischen Verfahrens (Tabelle 1-3). Insbesondere die Verwendung von Zink und Trimethylsilylchlorid (TMSCl) führte zu großen Abfallmengen mit einer sehr kostenintensiven Entsorgung.



(1) 3,4-Xylidin

(2) Schiffsche Base

(3) *N*-(3,4-dimethylphenyl)-D-1'-ribamin

(4) N-(2-phenylazo-4,5-dimethylphenyl)-D-1'-ribamin

(5) Barbitursäure

Abbildung 1-3: Syntheseschema Riboflavin (Vitamin B_2)

Ein weiteres Beispiel für den Erfolg biotechnologischer Verfahren ist die Produktion des Vitamin B_2 (Riboflavin), welches ursprünglich über einen komplexen

mehrstufigen vollsynthetischen Prozess dargestellt wurde (Abbildung 1-3) **[Bott *et al.* 2004]**. In diesem klassischen Ansatz sind mehrfach aufwendige Reaktionsbedingungen (z. Bsp. die Verwendung wasserfreier Lösungsmittel) nötig, was eine hohe Komplexität des Gesamtprozesses bewirkt und zu hohen Abfallmengen führt.

Dieses vollsynthetische Verfahren wurde bis 1990 durch einen einzigen biotechnologischen Arbeitsschritt ersetzt. Unter Verwendung von gentechnisch modifizierten Organismen (*Bacillus subtilis* oder *Ashbya gossypii*) konnte in einem einzigen Fermentationsschritt ausgehend von D-Glucose die Reaktionszeit und Abfallmenge deutlich reduziert werden. Weitere Prozessverbesserungen führten dazu, dass die Produktreinheit durch den biotechnologischen Prozess sogar noch erhöht werden konnte **[Transgen 2005]**.

2 Zielsetzung und Konzeption

Limitierungen verschiedenster Art stellen weiterhin ein Hauptproblem innerhalb der (industriellen) Biokatalyse dar und stehen oftmals effektiven Syntheseprozessen entgegen. Insbesondere durch die Verwendung nichtnatürlicher Substrate treten ungünstige Nebeneffekte auf **[Krishna 2002]**.

Die häufigsten Probleme sind:

- geringe Löslichkeit der beteiligten Substrate und Produkte
- ungünstige Gleichgewichtslage
- Nebenreaktionen
- mangelnde Stabilität des Biokatalysators
- Inhibierungen

Ziel dieser Arbeit ist es daher, verschiedene Konzepte der Einflussnahme auf die genannten Probleme zu untersuchen. Das Hauptaugenmerk soll auf dem Einsatz organischer Lösungsmittel liegen, welche für einige Enzymgruppen bereits erfolgreich eingesetzt werden (siehe dazu **Publikation 5**). Dieser Ansatz, auch bekannt als ‚Medium-Engineering', beinhaltet die Verwendung verschiedenster Reaktionskonzepte um mindestens eine der genannten Einschränkungen zu überwinden **[Vermue und Tramper 1995]**. Das Medium Engineering zielt auf die Beeinflussung der thermodynamischen und kinetischen Parameter, was u.a. die Grundlage für den Syntheseprozess bildet (Abbildung 2-1) **[Liese *et al.* 2006]**.

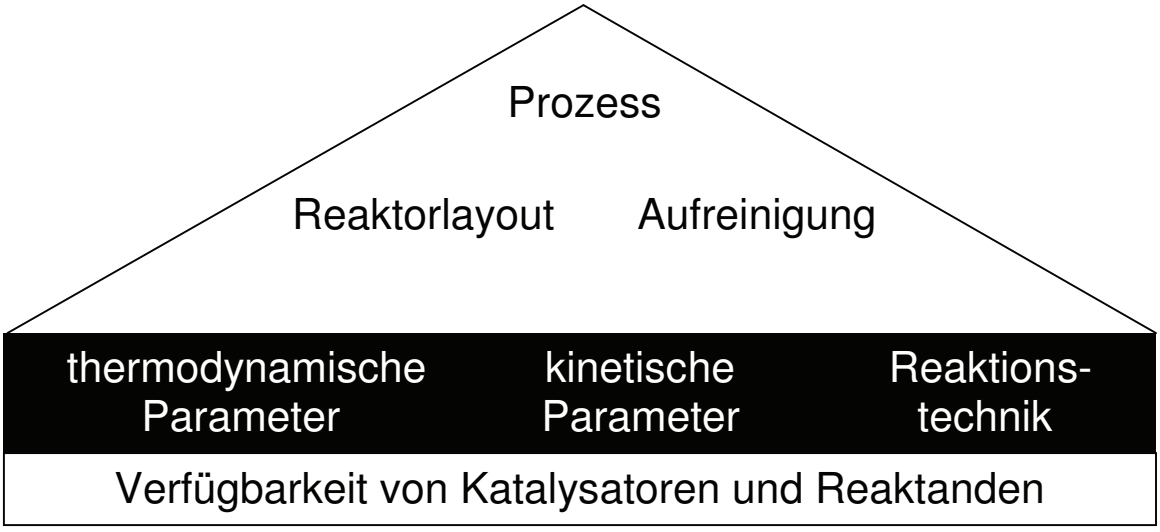


Abbildung 2-1: Allgemeines Schema des Prozessdesigns

Ausgehend von zwei Modellsystemen (Alkoholdehydrogenase- und Hydroxynitril Lyase katalysierte Reaktionen) mit spezifischen Problemstellungen sollen in der vorliegenden Arbeit die limitierenden Parameter des jeweiligen Syntheseprozesses identifiziert werden. Die Überwindung dieser Limitierungen soll mittels organischer Lösungsmittel mit dem Schwerpunkt des ‚Medium Engineering' erfolgen. Nach Auswahl der geeigneten Strategie sollen die Vorteile effektiv genutzt und (wenn möglich) die Ergebnisse in die Synthese (Gramm-Maßstab) übertragen werden (Abbildung 2-2).

Eine Übersicht über die in der vorliegenden Arbeit untersuchten Problemstellungen und Lösungsansätze ist in Tabelle 2-1 dargestellt. Eine Ausnahme stellt die **Publikation 5** dar, welche als Buchbeitrag eine Übersicht über den Bereich der nicht-wässrigen Enzymkatalyse beinhaltet.

Tabelle 2-1: Übersicht der Aufgabenbereiche

Enzymsystem	Problemstellung	Lösungsansatz	Publikationen
Hydroxynitril Lyase	Nebenreaktion	Modifizierung der Reaktionsparameter	2, 3, 4, 6
	ungünstige Gleichgewichtslage	Verzicht auf Cosolvent	2, 6
	geringe Löslichkeit	Zweiphasensystem	2, 3, 4, 6
Alkoholdehydrogenase	Substratinhibierung	Organisches Lösungsmittel als Cosolvents	1
Enzymklassen E.C. 1 – E.C. 6	Buchbeitrag zur Biokatalyse in nicht-wässrigen Medien		5

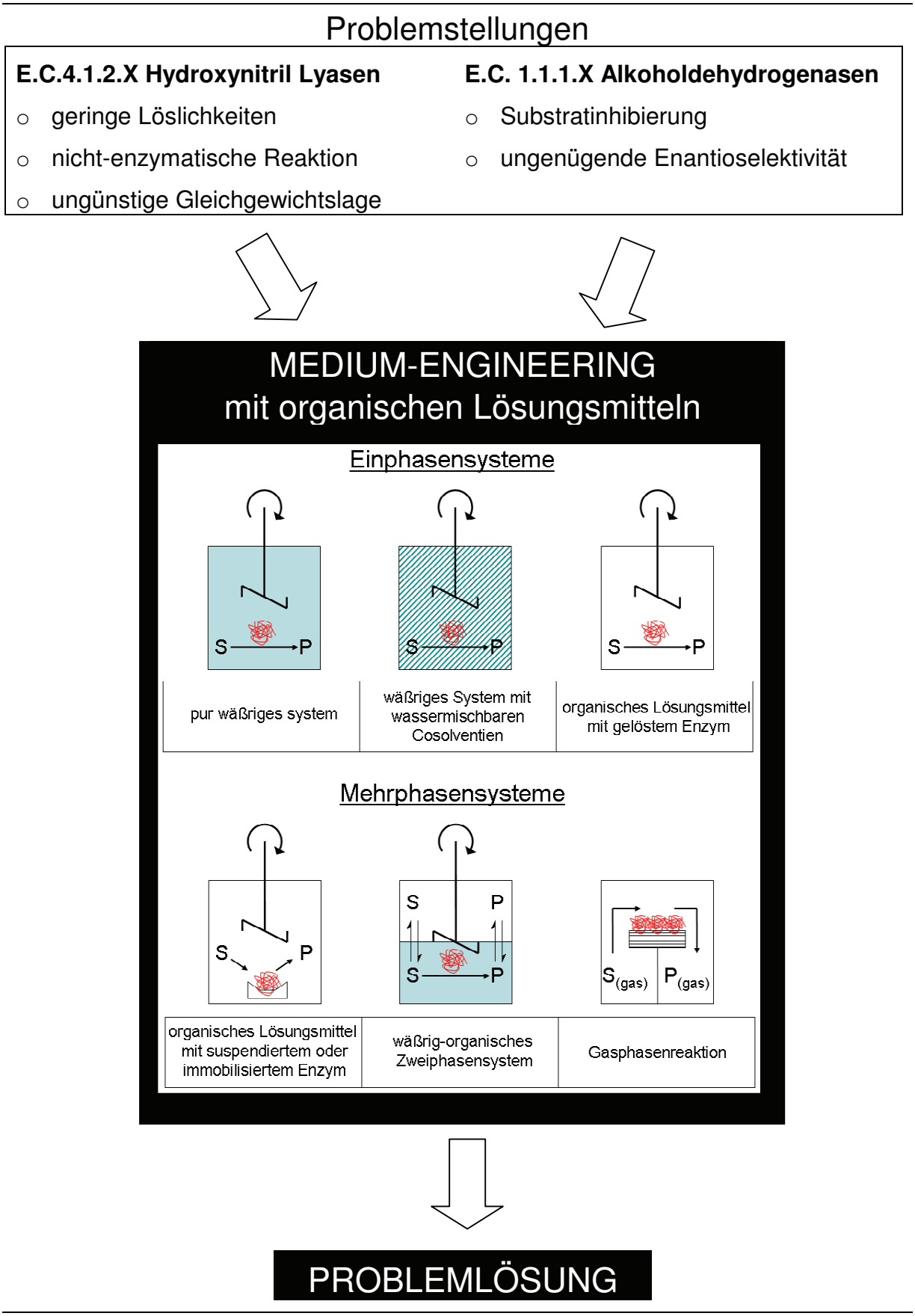


Abbildung 2-2: Allgemeine Zielstellung der Dissertation

3 Enzymkatalyse unter dem Einfluss organischer Lösungsmittel

3.1 Grundlagen

Enzyme katalysieren im Metabolismus der Mikroorganismen, Pilze, Pflanzen und Tiere verschiedenste Reaktionen, wobei sie durch ihre außerordentliche Stereo-, Regio- und Enantioselektivität im Allgemeinen nur für die Umsetzung eines oder nur weniger Substrate optimiert sind **[Löffler und Petrides 1998]**. Für den synthetischen Nutzen von Biokatalysatoren ist dagegen oftmals die Umsetzung von nicht-natürlichen Substraten relevant **[Schoemaker *et al.* 2003]**.

Günstigerweise weisen in zahlreichen Fällen natürliche Enzyme bereits schon solch ein breites Substratspektrum auf, welches zusätzlich noch durch die moderne Molekularbiologie verbessert werden kann (vgl.: Protein-Engineering). Andererseits besitzen zahlreiche Substanzen vom natürlichen Substrat deutlich abweichende Eigenschaften. Das wichtigste Kriterium ist in zahlreichen Fällen die sehr geringe Wasserlöslichkeit des entsprechenden Substrates. Somit wären prinzipiell sehr große wässrige Reaktionsvolumina notwendig, um das Produkt in größeren Maßstäben zu gewinnen, was bereits aus Kosten- und Effektivitätsgründen nicht möglich ist. Als logische Konsequenz werden an dieser Stelle Cosolventien zugesetzt, welche die Substratlöslichkeit anheben sollen **[Klibanov 2001]**. Cosolventien können ionische Flüssigkeiten, überkritische Medien oder organische Lösungsmittel sein, wobei die letzte Gruppe näher betrachtet werden sollen.

Prinzipiell lassen sich durch den Zusatz von organischen Lösungsmitteln Ein- als auch Mehrphasensystem ermöglichen (Abbildung 3-1) **[Carrea und Riva 2000]**.

Auf der einen Seite ergibt der Zusatz von wassermischbaren Cosolventien (z. Bsp. Acetonitril) einphasige wässrig-organische Reaktionssysteme. Hierbei verbleibt das Enzym in der wässrigen Phase, wobei das organische Lösungsmittel die Löslichkeit der schwerlöslichen Verbindung innerhalb des Einphasensystems anhebt. Das Gesamtsystem beinhaltet aber weiterhin einen überwiegenden Anteil von Wasser. Als Extremvariante sind rein organische Systeme möglich, welche in gelöster Form chemisch modifizierte Enzyme beinhalten **[DeSantis und Jones 1999, Salleh *et al.* 2002]**.

Auf der anderen Seite sind die Mehrphasensysteme durch mindestens 2 separate (Reaktions-)Zonen charakterisiert. Die unterschiedlichen Eigenschaften der verschiedenen Zonen ermöglicht nun die Verwendung gegensätzlicher chemischer und physikalischer Eigenschaften in einem Reaktionsgefäß (Kompartimentierung). Beispielsweise beinhaltet das wässrig-organische Zweiphasensystem eine wässrige Reaktionszone mit dem gelösten Enzym und eine organische Lösungsmittelphase mit den gelösten Substraten und, nach Abschluss der Reaktion, den Produkten. Dies vereinfacht deutlich die Abtrennung der Reaktanden und ermöglicht somit die Rezyklierung des Biokatalysators. Eine wichtige Variante ist hierbei der Einsatz micellarer Systeme, welche durch den Zusatz von amphiphilen Substanzen

zugänglich sind. Die somit erhaltenden Mikroemulsionen sind ebenso erfolgreich in der Biokatalyse eingesetzt worden **[Straathof 2003]**. Als Besonderheit ist die Gasphasenreaktion zu sehen, da hier das organische Lösungsmittel als ‚carrier-Gas' verwendet wird und nicht als flüssiges Lösungsmittel auftritt. Alternativ können auch Inertgase als ‚carrier-Gas' verwendet werden **[Ferloni *et al.* 2004]**.

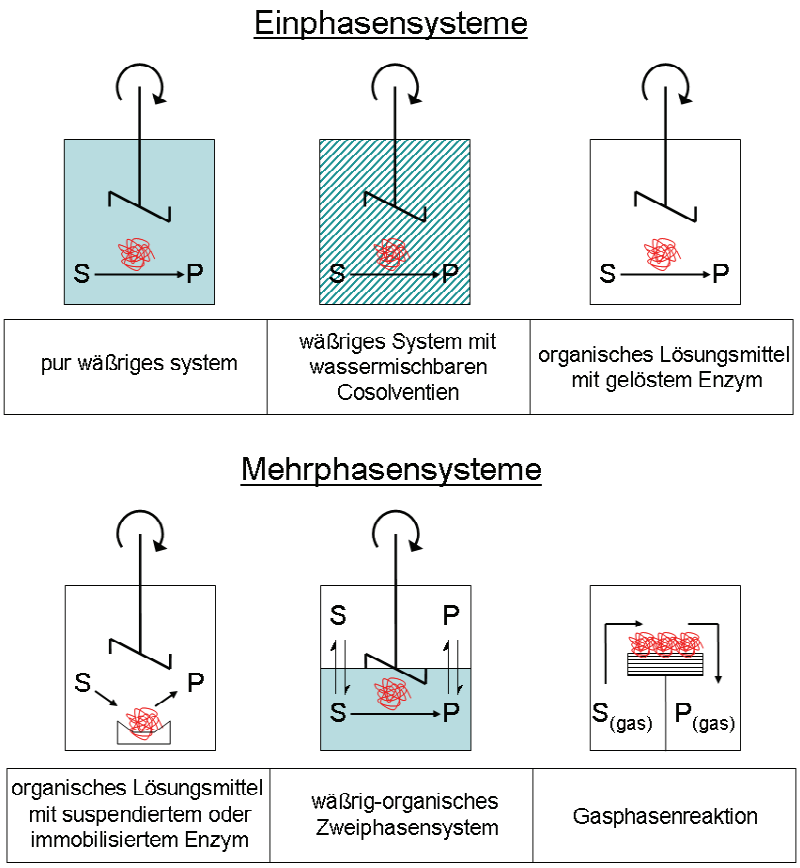


Abbildung 3-1: Übersicht der möglichen Phasensysteme (S – Substrat, P – Produkt)

Ein analoges Verhalten lässt sich in Teilen auch auf die weiteren Lösungsmittelvarianten, z. Bsp. Ionische Flüssigkeiten, übertragen **[Wasserscheid und Keim 2000]**. Diese neuartige Substanzklasse, dessen Vertreter tief schmelzende Salze sind, ermöglicht weitere Effekte, welchen insbesondere in den letzten beiden Dekaden eine hohe Aufmerksamkeit im Bereich der Biokatalyse widerfahren ist **[Klembt *et al.* 2007, van Rantwijk *et al.* 2003]**.

3.2 Beurteilungskonzepte für organische Lösungsmittel innerhalb der Biokatalyse

Der Vorteil organischer Lösungsmittel ist die außerordentlich große Bandbreite von Eigenschaften, welche durch die große Anzahl und Variationsbreite gegeben ist. Ihre Vielzahl an möglichen Strukturen begünstigt die Auswahl des optimalen

Reaktionsmediums. Im Vergleich zu Wasser ergeben sich die größten Variabilitäten im Bereich des Siedepunktes und der Viskosität (Tabelle 3-1). Der wichtigste Parameter ist an dieser Stelle aber sicherlich das Lösungsvermögen für hydrophobe Substrate und Produkte. Für die Produktivität eines chemischen Prozesses sind darüber hinaus weitere Faktoren wie die Flüchtigkeit des entsprechenden organischen Lösungsmittels wichtig.

Tabelle 3-1: Eigenschaften organischer Lösungsmittel

Lösungsmittel	molare Masse / $g \cdot mol^{-1}$	Siedepunkt / °C	Dichte / $g \cdot cm^{-3}$	Viskosität / mPa · s	logP/ -
Aceton	58.08	56	0.791	0.32	-0.23[2]
tert.-Butanol	74,14	83	0.79	3.3	0.8[3]
Ethanol	46.07	78	0.79	1.099	-0.24[2]
1,4-Dioxan	88.11	100	1.03	0.796[1]	-1.1[2]
MTBE	88.15	54	0.74	0.34	1.0[3]
n-Hexan	86.18	68	0.66	0.29	3.5[2]
2-Propanol	60.10	82	0.80	2.07	0.28[2]
Toluol	92.14	110	0.865	0.55	2.5[2]
Wasser	**18.01**	**100**	**1.0**	**1.0**	**-**

Quelle: Beilstein CrossFire Online (online-Zugriff der Universität Rostock); soweit nicht anders angegeben beziehen sich die Werte auf Raumtemperatur

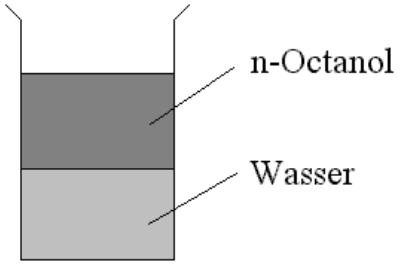
[1]48 °C; [2]entnommen aus: **[Laane *et al.* 1987]** (errechnet durch die Methode der hydrophoben Fragmentkonstanten; [3]entnommen aus: **[Filho *et al.* 2003]**

Dagegen ist für den Einsatz mit Biokatalysatoren an erster Stelle die Biokompatibilität der organischen Lösungsmittel relevant, da diese untereinander in Wechselwirkung stehen.

Zur Verallgemeinerung dieses Sachverhaltes wurden verschiedenste Konzepte zur Vorhersage der Biokompatibilität entwickelt.

<u>logP-Konzept</u>

Das klassische Konzept des logP-Wertes betrachtet direkt das Verhältnis der Hydophobizität zur katalytischen Aktivität des Biokatalysators im organischen Lösungsmittel. Der logP-Wert ist definiert als der dekadische Logarithmus des Verteilungskoeffizienten einer Substanz zwischen *n*-Octanol und Wasser (Abbildung 3-2).



$$\log P = \log\left(\frac{[S]_{n-OCTANOL}}{[S]_{WASSER}}\right)$$

P – Verteilungskoeffizient
[S] – Konzentration

Abb 3-2: Das logP-Konzept

Vereinfacht kann das logP-Konzept auch zur Vorhersage der Wasserlöslichkeit einer Verbindung verwendet werden (Tabelle 3-2) **[Laane *et al.* 1987]**.

Tabelle 3-2: Einteilung für die Enzymkatalyse und Wasserlöslichkeit mittels logP-Konzept

logP	Wassermischbarkeit	Effekt auf Enzymaktivität
logP < 0	vollständig wassermischbar	nur eingeschränkter Einsatz möglich, Zusätze von bis zu 10 Vol% werden üblicherweise ohne Enzymdesaktivierung toleriert
0 < logP < 2	teilweise wassermischbar	kaum Einsatzmöglichkeiten, da die Enzym-desaktivierung schnell einsetzt
2 < logP < 4	nur geringe Wassermischbarkeit	keine allgemeine Aussage über die Einsatz-möglichkeiten möglich
logP > 4	nicht wassermischbar	sehr hydrophob, Enzymkatalyse ist meist uneingeschränkt möglich

Der in der deutschen Literatur auch als K_{ow}–Wert bezeichnete Verteilungskoeffizient zwischen *n*-Octanol und Wasser ist ein Maß für die Hydrophobizität einer Verbindung, ein hoher logP-Wert steht für eine hohe Hydrophobizität **[Vermue und Tramper 1995]**. Eine weitere Methode zur Bestimmung des logP-Wertes ist die Berechnung aus „hydrophoben Fragmentkonstanten“ (hydrophobic fragmental constants) und ähnlichen Verfahren **[Sakuratani *et al.* 2007]**. Durch das serienmäßige Vermessen der logP-Werte homologer Verbindungsreihen konnte der einzelne Effekt einer funktionellen Gruppe auf den logP-Wert ermittelt und somit rechnerisch der Verteilungskoeffizient zwischen *n*-Octanol und Wasser bestimmt werden.

Schlussendlich zeigt das logP-Konzept in erster Näherung eine Übereinstimmung zwischen steigendem logP-Wert und steigender Enzymaktivität (Abbildung 3-3). Dagegen wurde beobachtet, dass in einigen Fällen dieses Prinzip nicht auf alle Biokatalysatoren übertragbar ist. Die Hauptgründe sind an dieser Stelle spezifische, nicht allein durch globale Eigenschaften des Moleküls definierte, Wechselwirkungen

zwischen den Lösungsmittelmolekülen und dem Enzym. Mögliche sekundäre Effekte sind:

- kompetetive Inhibierung des Enzyms
- Ausbildung einer nicht aktiven Spezies durch inkorrekte Protein-Faltung (z. Bsp. durch eine Neuordnung von Wasserstoffbrückenbindungen)
- irreversible Proteinfällung
- direkte chemische Reaktion zwischen Enzym und Lösungsmittelmolekül

Dementsprechend werden auch regelmäßig Abweichungen von diesem Verhalten beschrieben (siehe auch: Pankreas Lipase, Abbildung 3-3) **[Filho *et al.* 2003]**.

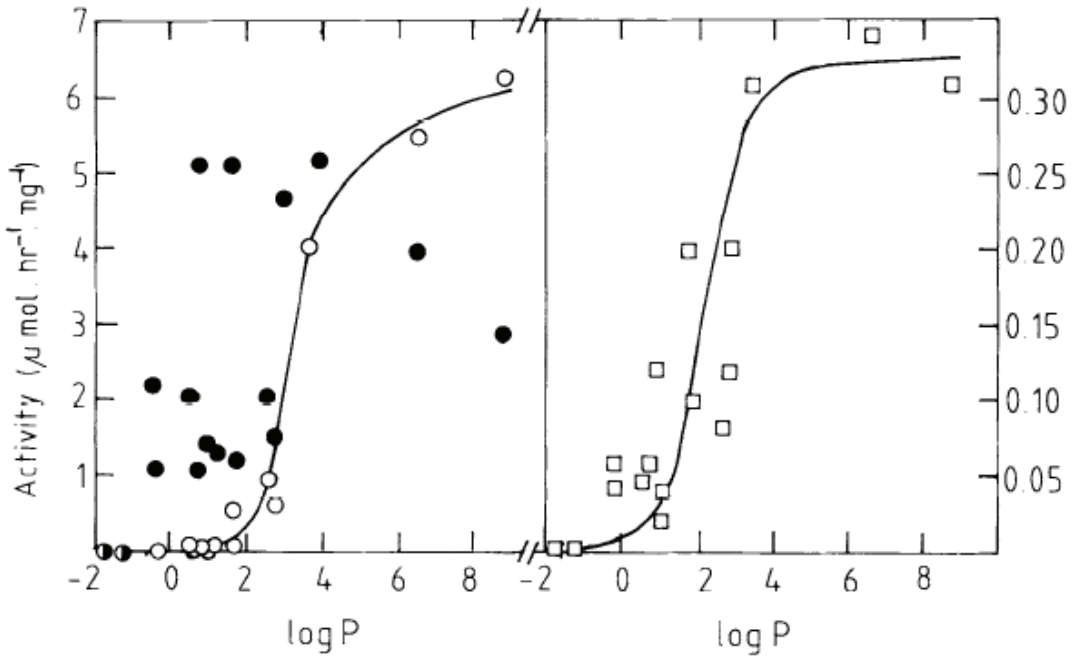


Figure 2. Initial rates of lipase-catalyzed transesterification reaction between tributyrin and heptanol in different, nearly anhydrous organic solvents vs. log *P*. Activity data were taken from ref. 2. Log *P* values were calculated from hydrophobic fragmental constants according to refs. 6 and 7. (●) Pancreatic lipase. (○) Yeast lipase. (□) Mold lipase.

Abbildung 3-3: Beziehung zwischen logP-Wert und Enzymaktivität **[Laane et al. 1987]**

Zusammenfassend muss gesagt werden, dass das logP-Konzept nur in einem sehr allgemeinen Rahmen gültig ist, z. Bsp. beim Vergleich von Substanzen der gleichen Stoffgruppe **[Filho *et al.* 2003]**. Die Biokompatibilität des entsprechenden Lösungsmittels kann auf Grund oben genannten Gründe prinzipiell nicht durch eine einzige globale Eigenschaft (Hydrophobizität) beschrieben werden.

Sehr gut lassen sich die Grenzen des logP-Konzeptes am Beispiel von *tert.*-Butanol darstellen. Auf der einen Seite ermöglicht die *tertiär*-Butylgruppe durch ihre hohe Hydophobizität eine gute Verträglichkeit mit Enzymen (siehe auch Tabelle 3-2). Auf

der anderen Seite stellt die Hydroxylgruppe eine sehr hydrophile Endgruppe dar, die ausreichend hohe Wasserlöslichkeiten zulässt. Dementsprechend besitzt *tert.*-Butanol einen logP-Wert von 0.8, was nach der Einteilung von Laane *et al.* eine schnelle Enzymdeaktivierung erwarten lässt. Interessanterweise stellt *tert.*-Butanol nun ein gutes Lösungsmittel für die Enzymkatalyse dar, was auf eine deutlichere Wirkung der hydrophoben Endgruppe schließen lässt.

Daraus folgert, dass das logP-Konzept durchaus in erster Näherung eine Aussage zur Biokompatibilität eines organischen Lösungsmittel ermöglicht. Aber auf Grund von zahlreichen spezifischen Wechselwirkungen (siehe *tert.*-Butanol) ist keine direkte Korrelation zwischen Hydrophizität und Enzymaktivität zu erreichen. Vielmehr ist das logP-Konzept nur als ein Indiz bei der Beurteilung von nicht-wässrigen Reaktionskonzepten zu verstehen.

Wasseraktivität

Ein alternatives Konzept stellt die Ermittlung der Wasseraktivität im Verhältnis zur Enzymaktivität dar, welches somit nicht direkt an die physikalischen Eigenschaften des entsprechenden Lösungsmittels gekoppelt ist. Entgegengesetzt zum logP-Konzept wird vielmehr das Wasser im organischen Lösungsmittel betrachtet, mit der Einschränkung, dass nur die Wirkung des Wassers betrachtet wird **[Halling 1994]**.

Die Wasseraktivität ist definiert als **[Brdička 1982]**:

$$a_{H_2O} = \frac{f_{H_2O}}{f^*_{H_2O}}$$

f_i – Fugazität von Wasser in Mischung; f_i^* – Fugazität von reinem Wasser

Die Fugazität kann gegen den Partialdruck ausgetauscht werden **[Atkins 1996]**

$$\boxed{a_{H_2O} = \frac{p_{H_2O}}{p^*_{H_2O}}}$$

p_{H2O} – Partialdruck von Wasser über der Mischung;
p_{H2O}^* – Dampfdruck von reinem Wasser

Die verschiedenen organischen Lösungsmittel haben unterschiedliche Einflüsse auf die Wasseraktivität. So weist Acetonitril auch bei einem niedrigen Molenbruch von Wasser eine sehr viel höhere Wasseraktivität auf als beispielsweise DMSO (Abbildung 3-4). Die Differenzen erklären sich durch die unterschiedliche Affinität der Lösungsmittel zum Wasser, z. Bsp. durch die Varianz der Solvathüllen der verschiedenen Lösungsmittelmoleküle.

Für den Einsatz innerhalb der Biokatalyse ergibt sich, dass vorzugsweise hohe Wasseraktivitäten notwendig sind, um die katalytische Aktivität des Biokatalysators zu gewährleisen. So sind im vorliegenden Fall für Dimethylsulfoxid (DMSO), im Vergleich zu Acetonitril, für hohe Wasseraktivitäten deutlich höhere Konzentrationen an Wasser notwendig. Im Umkehrschluss bedeutet es, dass große Zusätze von Acetonitril nur einen geringen Einfluss auf die Wasseraktivität haben und somit als Cosolvents deutlich besser geeignet sind. Weiterhin bildet an dieser Stelle die Gruppe der Lipasen eine Ausnahme, welche auch bei sehr geringen Wassergehalten und Wasseraktivitäten außerordentlich hohe Enzymaktivitäten aufweisen. Darauf begründet sich auch die große Anzahl an Anwendungen der Lipasen in organischen Lösungsmitteln (siehe **Publikation 5**).

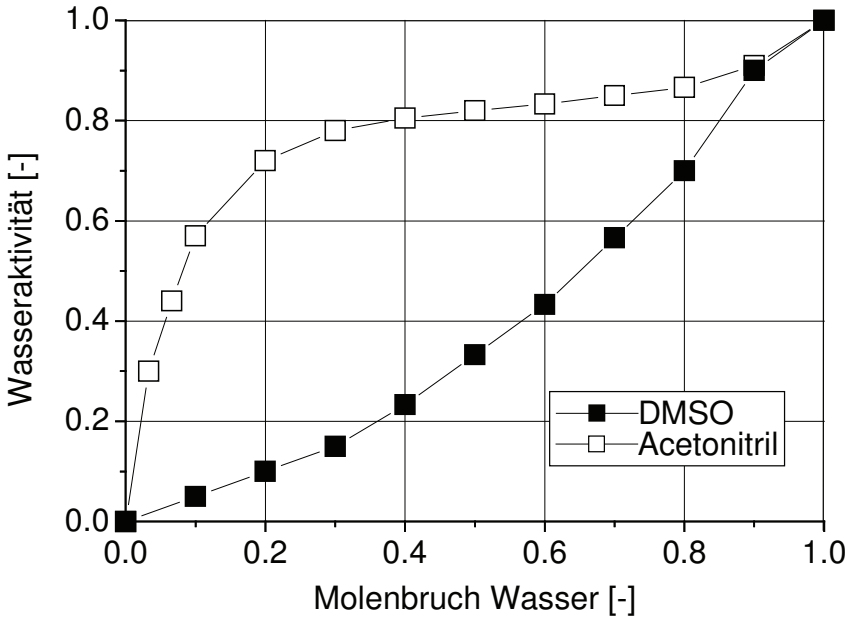


Abb. 3-4: Wasseraktivität vs. Molenbruch Wasser für DMSO und Acetonitril **[Bell et al. 1997]**

Nichtsdestotrotz ist das Modell der Wasseraktivität nicht ohne weitere Einschränkungen nutzbar um biokatalytische Reaktionen im Beisein von organischen Lösungsmitteln zu beschreiben. Beispielsweise sind im Bereich der Alkoholdehydrogenasen auch deutlich unterschiedliche Reaktivitäten bei vergleichbaren Wasseraktivitäten zu beobachten (Abbildung 3-5) (siehe auch **Publikation 1**) **[Wehtje *et al.* 1997]**.

Die Ursache ist analog zum logP-Konzept erneut die Tatsache, dass neben den globalen Eigenschaften spezielle Wechselwirkungen zwischen dem organischen Lösungsmittel und dem Protein auftreten. Diese sekundäre Einflussfaktoren sind mit

dem Konzept der Wasseraktivität nicht zu beschreiben und limitieren die Anwendbarkeit dieses Konzeptes.

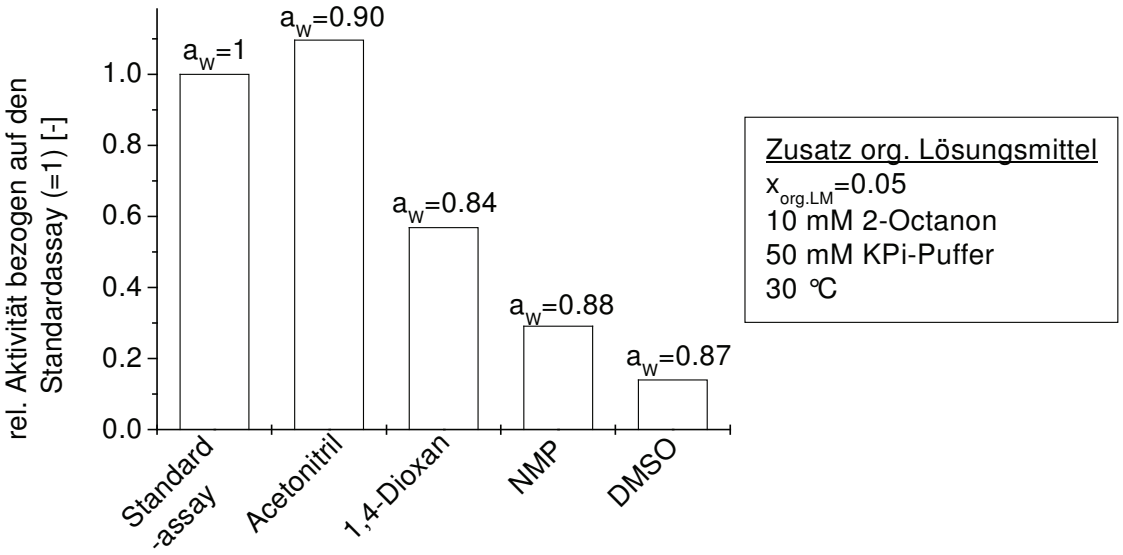


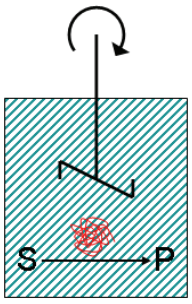
Abbildung 3-5: Vergleich Wasseraktivität vs. Enzymaktivität am Beispiel einer Alkoholdehydrogenase **[Schumacher 2004]**

Neben den vorgestellten Konzepten sind noch weitere Parameter untersucht worden, welche andere physikalisch-chemische Eigenschaften zur Beurteilung der biokatalytischen Aktivität betrachten. Beispielsweise kann die Polarität einer Verbindung über den Dimroth-Reichardt-Parameter (E_T30) angegeben werden, welcher ebenfalls rudimentär Aussagen zur Biokompatibilität ermöglicht **[Torres *et al.* 1998]**. In Analogie zu den vorhergehenden Beispielen wird auch hierbei eine globale Eigenschaft des Lösungsmittelmoleküls untersucht und weitere Wechselwirkungen zwischen Biokatalysator und des organischen Lösungsmittelmoleküls nicht betrachtet.

Eine übergreifende Beurteilung ergibt an dieser Stelle, dass zur Zeit kein praktikables System zur Beurteilung von organischen Lösungsmittel für die Biokatalyse existiert. In erster Näherung ermöglichen die dargestellten klassischen Konzepte Vorhersagen, die aber immer von der Struktur und spezifischen Wechselwirkungen des organischen Lösungsmittels verfälscht werden.

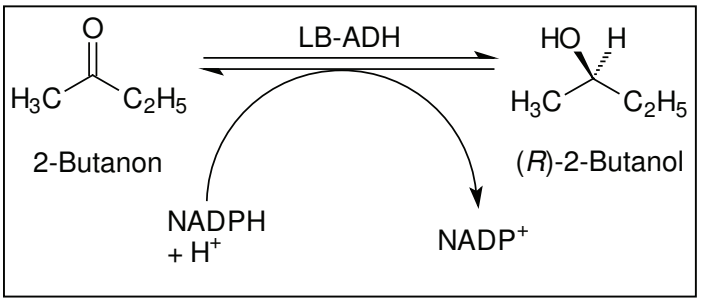
Vielmehr ist weiterhin das Experiment notwendig, um die Biokompatibilität eines organischen Lösungsmittels zu untersuchen und einen möglichen Einsatzzweck abzuschätzen.

3.3 Wässrig-organisches Einphasensystem



wäßriges System mit wassermischbaren Cosolventien

Für die Gruppe der wässrig-organischen Einphasensysteme werden üblicherweise Lösungsmittel mit einem logP-Wert < 0 verwendet **[Castro und Knubovets 2003, Dordick 1989]**. In einigen Grenzfällen kann der Bereich, mit eingeschränkter Mischbarkeit, bis zu einem logP-Wert = 1.0 aufgeweitet werden. Für die Verwendung der Einphasensysteme sind Konzentrationen von bis zu 10% (v/v) organisches Lösungsmittel üblich, welche normalerweise vom Enzym ohne Probleme toleriert werden. Nur in wenigen Ausnahmefällen sind Konzentrationen bis zu 70% (v/v) möglich **[Faber 2004]**. Das Hauptziel des wässrig-organischen Einphasensystem liegt dabei üblicherweise in der Löslichkeitserhöhung für ein wenig wasserlösliches Substrat. Weiterhin lassen sich kinetische Parameter einer Reaktion manipulieren. So konnte beispielsweise anhand der Alkoholdehydrogenase aus *Lactobacillus brevis* (LB-ADH) eine Substratinhibierung durch die Beigabe von wassermischbaren organischen Lösungsmitteln (z. Bsp. Acetonitril) komplett beseitigt werden (Abbildung 3-6) (**Publikation 1**).



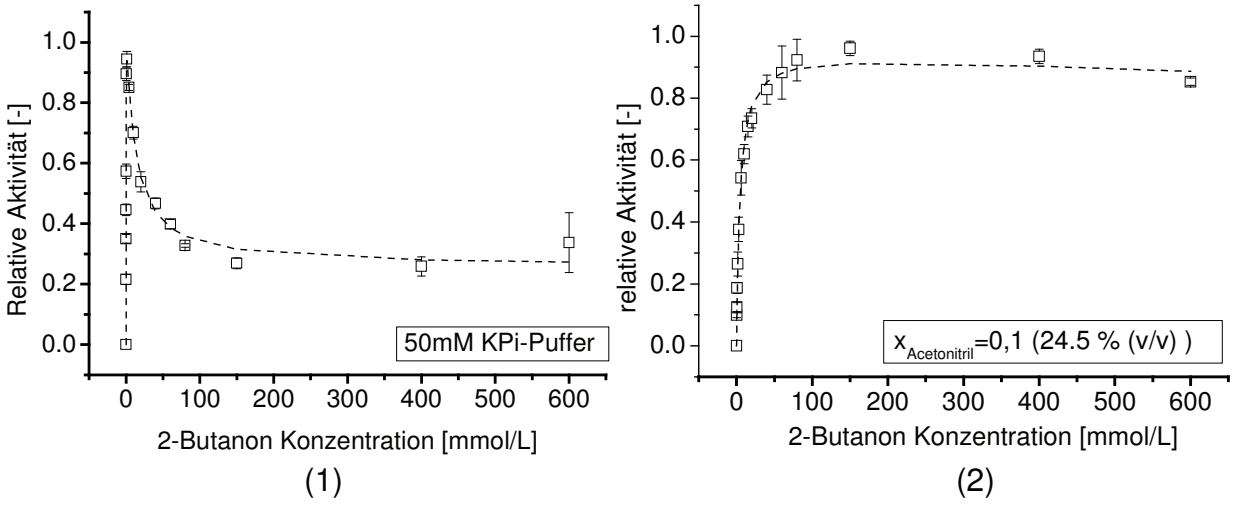


Abbildung 3-6: Beeinflussung kinetischer Parameter am Beispiel der LB-ADH

Während in reinem Puffer (1) noch eine deutliche Substratinhibierung sichtbar ist, so kann durch die Zugabe von 24.5 % (v/v) Acetonitril ($x_{Acetonitril}$0.1) die Inhibierung komplett aufgehoben werden (2). Begründet wird dieses Verhalten durch eine Zunahme der Hydrophobizität innerhalb der bulk-Phase, so dass die Affinität der LB-ADH zum Substrat künstlich verringert wird. Dies lässt sich ebenso gut am steigenden K_M-Wert erkennen.

Eine weitere Möglichkeit ist der Einsatz wassermischbarer Lösungsmittel zur systematischen Überwindung thermodynamischer Limitierungen. Am Beispiel von Hydroxynitril Lyase katalysierten Reaktionen konnte gezeigt werden, dass durch die Erhöhung der Löslichkeit eines Substrates der Gleichgewichtsumsatz zu Gunsten höherer Umsätze beeinflusst werden kann (Abbildung 3-7) (**Publikation 2**). Dieses Grundlagenwissen ermöglichte im folgenden Arbeitsschritt die Verwendung lösungsmittelfreier Zweiphasensysteme, wobei die Gleichgewichtsumsätze erneut deutlich gesteigert werden konnten.

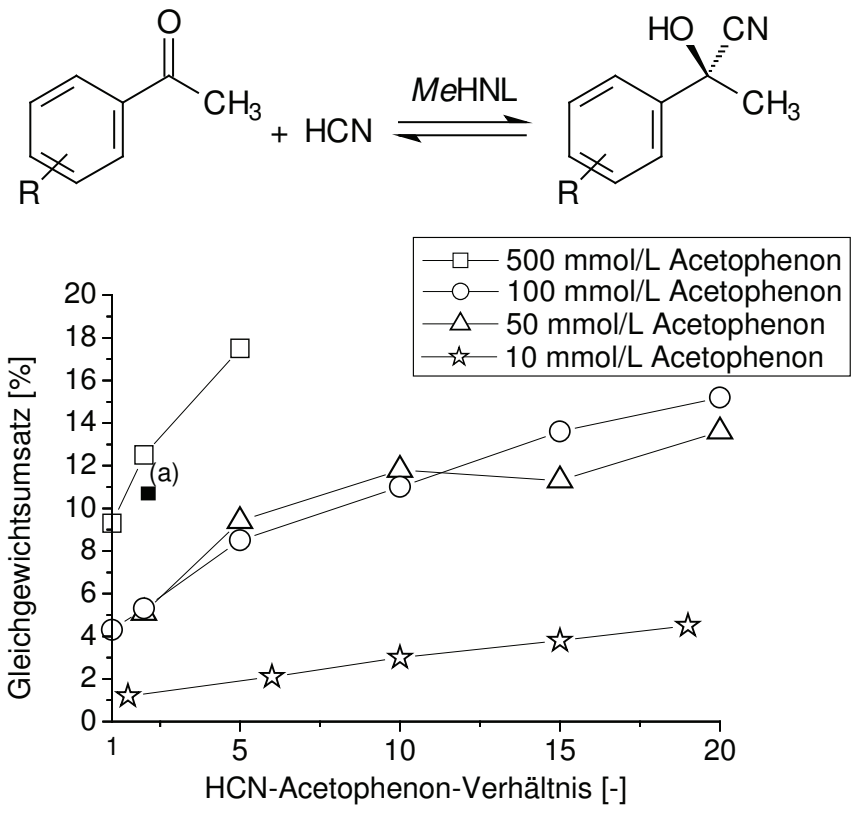


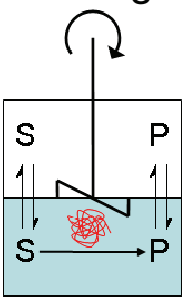
Abbildung 3-7: Überwindung thermodynamischer Limitierungen durch wässrig-organische Einphasensysteme

Cosolvent Ethanol: 0% (v/v) für 10 mmol/L Acetophenon, 25% (v/v) für 50 mmol/L Acetophenon, 35% (v/v) für 100 mmol/L Acetophenon, 50% (v/v) für 500 mmol/L Acetophenon;(a) **[Bühler *et al.* 2003]**

Durch die Besonderheit des Reaktionssystems, dass aus 2 Molekülen Substrat nur 1 Molekül Produkt entsteht, sind bei höheren Startkonzentrationen des Substrates höhere Gleichgewichtsumsätze möglich (Entropieeffekt). Durch die

löslichkeitsvermittelnde Eigenschaft des Ethanol wird hierbei erst eine ausreichend hohe Löslichkeit erreicht. Dieser Effekt lässt sich durch eine weitere *in situ* Extraktion steigern, wobei aber zusätzlich eine Substratphase (Zweiphasensystem) notwendig ist (**Publikation 2 & 6**).

3.4 Wässrig-organisches Zweiphasensystem



wäßrig-organisches Zweiphasensystem

Im Gegensatz zum wässrig-organischen Einphasensystem kann durch den Einsatz von nicht-wassermischbaren organischen Lösungsmitteln ein Zweiphasensystem erhalten werden (logP > 1.0). Hierdurch tritt direkt eine Kompartimentierung ein, welche mindestens zwei Zonen bildet. Üblicherweise stellt die Phase des organischen Lösungsmittels ein Reservoir dar, worin die Substrate und Produkte gelöst sind. Dagegen liegen in der wässrigen Phase (in seltenen Fällen auch direkt an der Grenzfläche **[Hickel *et al.* 1999]**) die Enzyme vor. Durch die geringe Wasserlöslichkeit wird die Wasseraktivität in der wässrigen Phase nur gering herabgesetzt. Prinzipiell kommt es an dieser Stelle nur auf die Biokompatibilität des eingesetzten Lösungsmittels an, wobei logP-Werte > 4 im Allgemeinen nur einen geringen negativen Effekt auf die biokatalytische Reaktion haben.

Dieser experimentelle Ansatz hat verschiedene Vorteile:

1. Die hohen Verteilungskoeffizienten der Substrate und Produkte erwirken eine Anreicherung der Reaktanden in der organischen Phase, was somit die Produktgewinnung vereinfacht. Entgegengesetzt dazu kann die wässrige Phase mit dem gelösten Biokatalysator mehrfach wieder recycelt werden.
2. Wenn ein höherer Verteilungskoeffizient für das Produkt als für das Substrat auftritt, dann erfolgt eine selektive Extraktion aus der wässrigen Phase. Diese zusätzliche thermodynamische Triebkraft ermöglicht schlussendlich höhere Produktkonzentrationen in der organischen Phase, wobei das chemische Gleichgewicht in der wässrigen Phase natürlich nicht beeinflusst wird.
3. Im Falle von hohen Verteilungskoeffizienten liegen in der wässrigen Phase nur äußerst geringe Konzentrationen der Reaktanden vor. Somit sind Substrat- und Produktinhibierungen praktisch zu vernachlässigen. Gleichzeitig kann die Substratkonzentration in der wässrigen Phase auch unter den entsprechenden K_M-Wert sinken, so dass nur noch geringe Reaktionsgeschwindigkeiten auftreten.

Für den Bereich der Hydroxynitril Lyase katalysierten Cyanhydrinsynthese gibt es zusätzlich noch einen weiteren Vorteil, welcher aus dem Punkt 3 folgt. Da die nicht-enzymatische Reaktion direkt an die Substratkonzentration in der wässrigen Phase

gekoppelt ist, ergibt sich im Zweiphasensystem somit gleichzeitig eine Reduktion dieser ungewollten Nebenreaktion. Dies ermöglicht eine Erhöhung des e.e.-Wertes des Produktes **[Gerrits *et al.* 2001]**.

Eine Sonderform des Zweiphasensystems wurde in den **Publikationen 2 & 6** betrachtet. Hierbei konnte die organischen Phase direkt gegen eine reine Substratphase (Acetophenon) ausgetauscht und damit ein verändertes Extraktionsgleichgewicht erreicht werden. Während in der wässrigen Phase die volle Substratlöslichkeit erreicht wird (siehe auch Kapitel 3.3), stellt sich für das Produkt (Acetophenoncyanhydrin) ein großer Verteilungskoeffizient ein. Die daraus resultierende zusätzliche Extraktion des Produktes aus der wässrigen Phase verschiebt das Gleichgewicht hin zu höheren Umsätzen (Abbildung 3-8). Zusätzlich ermöglicht die hohe Acetophenonkonzentration eine hohe Enzymaktivtät, da keine Substratinhibierung auftritt. Dies resultiert wiederum in einer höheren Reaktivität und geringeren Reaktionszeiten. Die mäßige Hydrophobizität von Acetophenon (logP = 1.8) wirkt sich dagegen negativ auf die Enzymstabilität aus. Dies konnte aber ausreichend durch eine Erhöhung der Enzymkonzentration ausglichen werden.

Durch die Verwendung dieses lösungsmittelfreien Zweiphasensystems konnten Umsätze bis zu 36 % erreicht werden, obwohl der Gleichgewichtsumsatz im konventionellen Zweiphasensystem (mit Diisopropylether) bei nur 2.5 % liegt. Darüber hinaus lässt sich dieses Reaktionsprinzip problemlos auf weitere Substrate übertragen, z. Bsp. 2'-Fluor-acetophenon mit einem Umsatz von 71% und e.e.(*S*) > 99 %.

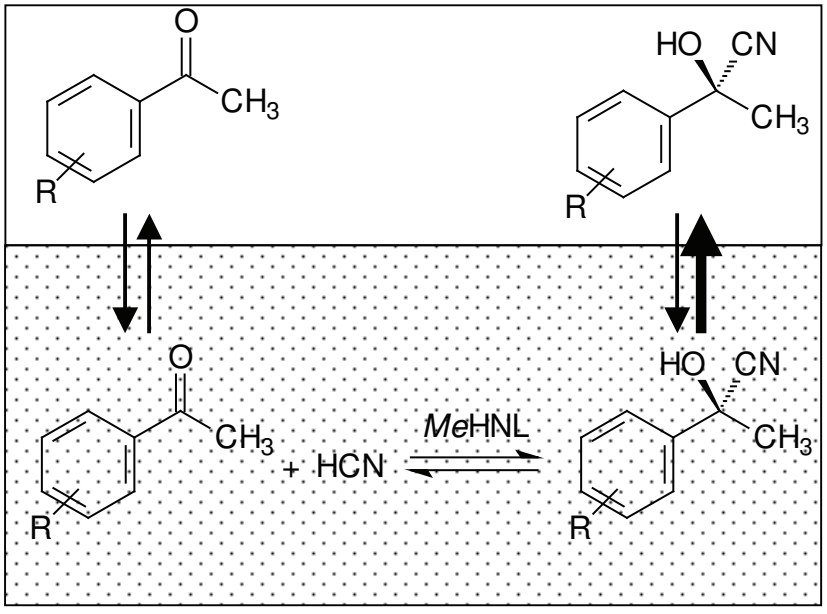


Abbildung 3-8: Lösungsmittelfreies Zweiphasensystem – Acetophenon/Puffer

Außerdem konnten 5 ml enantiomerenreines Acetophenoncyanhydrin hergestellt werden. Interessanterweise lässt sich dieses Produkt entgegen der allgemeinen Instabilität der Cyanhydrine ohne Zerfall und Racemisierung im Hochvakuum destillieren.

4 Alkoholdehydrogenasen

4.1 Einleitung sekundäre Alkohole

Die Synthese von enantiomerenreinen sekundären Alkoholen wurde bereits anhand von Biokatalysatoren und auch Übergangsmetallkatalysatoren mit verschiedenen Reaktionskonzepten untersucht **[Goldberg *et al.* 2007(a), Goldberg *et al.* 2007(b), Kroutil *et al.* 2004, Muller *et al.* 2005]**. Prinzipiell sind sekundäre Alkohole über zwei separate Wege zugänglich, die enantioselektive Reduktion (bzw. Hydrierung) und die Racematspaltung (Abbildung 4-1).

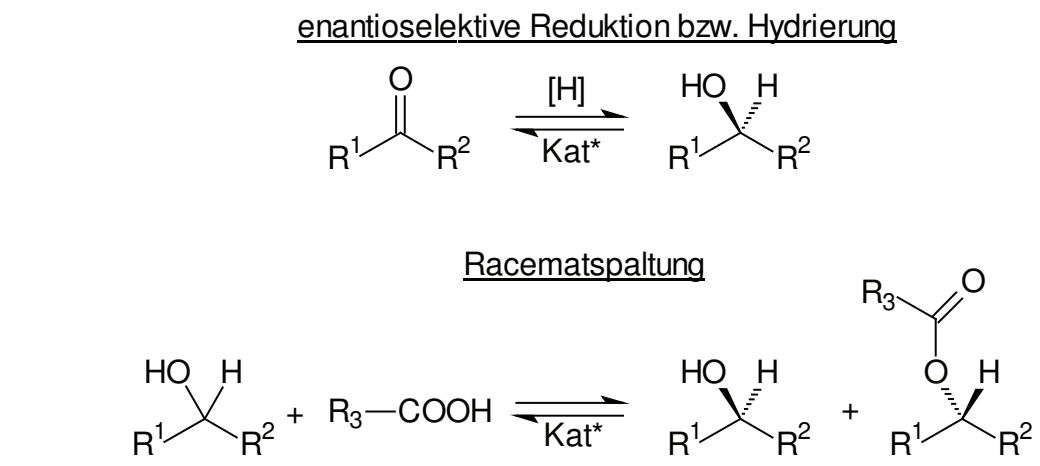


Abbildung 4-1: Mögliche Synthesemöglichkeiten für sekundäre Alkohole
Kat* = Biokatalysator bzw. Übergangsmetallkatalysator;

Auf der Seite der Biokatalyse wird die enantioselektive Reduktion größtenteils durch Alkoholdehydrogenasen abgedeckt, während in der chemischen Katalyse Metallkatalysatoren zum Einsatz kommen (z. Bsp.: Ru, Rh, Ir und Fe) **[Noyori 2002]**. Racematspaltungen werden biokatalytisch mit Lipasen und metallkatalytisch mit Ru- und Pd-Komplexen durchgeführt **[Gotor-Fernandez *et al.* 2006, Pellissier 2008]**.

Der direkte Vergleich dieser beiden Methoden zeigt, dass die Reduktion von prochiralen Substanzen zu sekundären Alkoholen deutlich effektiver ist, da in einem Reaktionsschritt hohe Enantiomerenüberschüsse und Umsätze bis zu 100% möglich sind. Im Gegensatz dazu gestattet die Verwendung einer Racematspaltung im Idealfall nur Umsätze von 50%, da ein Enantiomer nicht umgesetzt wird. Dieser Nachteil kann über den Umweg einer dynamisch kinetischen Racematspaltung überwunden werden, wobei das nicht umgesetzte Substrat *in situ* racemisiert und Umsätze von bis zu 100% erhältlich sind **[Pellissier 2008]**. Dies kann auch mit einer Kombination von Metallkatalysatoren und Enzymen erfolgen **[Martin-Matute und Backvall 2007]**.

Schlussendlich hat sich auf Grund der simplen Reaktionsführung größtenteils die enantioselektive Reduktion als Synthesevariante durchgesetzt **[Daussmann 2005]**. Die entsprechend dargestellten sekundären Alkohole stellen wichtige Intermediate (building blocks) für eine Vielzahl von Pharmazeutikar dar, z. Bsp.: für den HIV-Protease-Inhibitor Atazanavir **[Patel 2004]** und die cholesterinsenkende Substanz Atorvastatin **[Muller 2005]** (Abbildung 4-2).

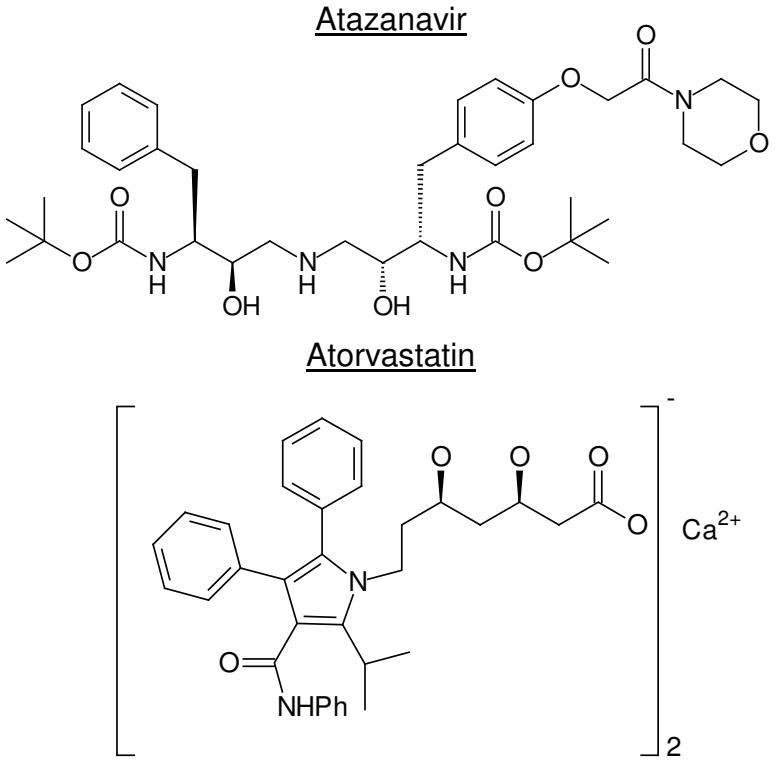


Abbildung 4-2: Atazanavir und Atorvastatin

4.2 Enzymatische Reduktion von prochiralen Ketonen

Die ADH's gehören zur Gruppe der Oxidoreduktasen (E.C. 1) und ihre natürliche Funktion liegt in der reversiblen Reduktion (Hydrierung) von Aldehyden und Ketonen zu den entsprechenden Alkoholen **[Drauz und Waldmann 2002]** (Abbildung 4-3).

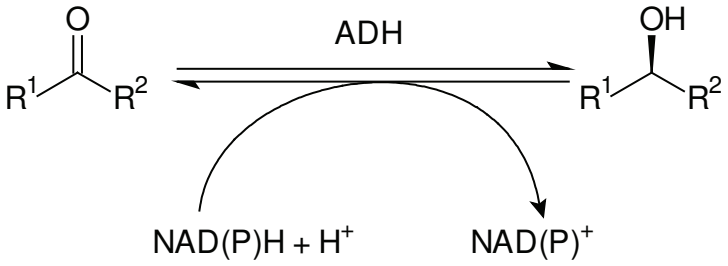


Abbildung 4-3: Enzymatische Reduktion von Aldehyden und Ketonen

Natürliche Reaktionen sind beispielsweise in der Glykolyse die Reduktion von Glycerinaldehyd zu Glycerin und als Oxidationsreaktion die Umsetzung von Ethanol zu Acetaldehyd **[Löffler und Petrides 1998]**. Als Besonderheit entsteht bei der Reduktion von unsymmetrischen Ketonen ein chirales Produkt **[Kataoka *et al.* 2003]**.

Die Reduktion mit Alkoholdehydrogenasen erfordert die Verwendung eines zusätzlichen Cofaktors: NAD(P)H. Dieser geht in den katalytischen Zyklus als Überträger von Redoxäquivalenten ein und wird während der Reduktionsreaktion zu $NAD(P)^+$ oxidiert (Abbildung 4-4).

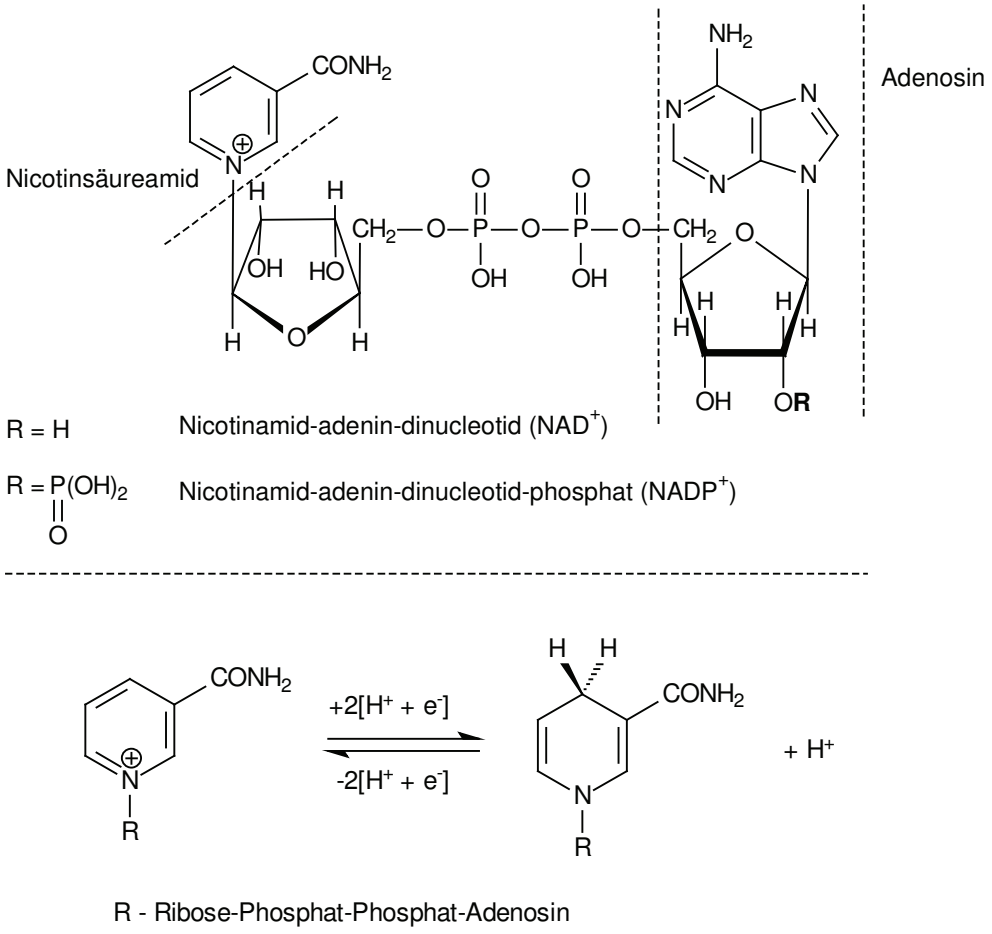


Abb. 4-4: Oxidierte Form des Cofaktors - $NAD(P)^+$

Ungünstigerweise lässt der hohe Preis des Cofaktors (NAD(P)H) keinen quantitativen Einsatz zu, so dass dieser *in situ* regeneriert werden muss. Aus diesem Grund wurden verschiedene Verfahren zur Cofaktorregenerierung entwickelt (Tabelle 4-1) **[Adlercreutz 1996, Eckstein *et al.* 2004(a), Stillger *et al.* 2002]**.

Tabelle 4-1: Möglichkeiten der Cofaktorregenerierung

Regenerierungsmethode	Erläuterung
substratgekoppelte Cofaktorregenerierung	Der enzymatischen Reaktion wird ein Cosubstrat hinzugesetzt, welches, unter Gewinnung des regenerierten Cofaktors, von der gleichen Alkoholdehydrogenase umgesetzt wird. Das Cosubstrat dient in der Reduktionsreaktion als Wasserstoffdonor und in der Oxidationsreaktion als Wasserstoffakzeptor.
	Beispiel: Isopropanol (Reduktionsreaktion) bzw. Aceton (Oxidationsreaktion)
enzymgekoppelte Cofaktorregenerierung	Bei der enzymgekoppelten Cofaktorregenerierung erfolgt dieser zusätzliche Teilschritt mit einem anderen Enzymkomplex mit einem separaten Cofaktor. Besonders effektiv sind hierbei Regenerierungsreaktionen mit einem irreversiblen Teilschritt, da sie nahezu vollständige Umsätze ermöglichen.
	Beispiele: Formiat und Formiatdehydrogenase; Glucose und Glucosedehydrogenase (beide in der Reduktionsreaktion)
Chemische oder metall-katalytische Verfahren	Neben der klassischen Reduktion von $NAD(P)^+$ mit Dithionit wurden zahlreiche weitere Verfahren basierend auf Übergangsmetallkatalysatoren (üblicherweise Rhodium-Katalyse) entwickelt. Neben der direkten Hydrierung mit Cosubstraten sind weitere auf elektrochemischen Verfahren basierende Prozesse bekannt **[Steckhan *et al.* 1990]**. Prinzipiell ähneln diese Verfahren den enzymatischen Prozessen.

4.3 Stand der Technik

Die Alkoholdehydrogenasen gehören zu der Gruppe von Enzymen, die bereits erfolgreich mit einer Vielzahl von organischen Lösungsmitteln eingesetzt wurden **[Gröger *et al.* 2003, Hummel 1999, Hummel *et al.* 2003]**. Neben dem Einsatz als freies Enzym ist ebenso die Möglichkeit der Verwendung in der Ganzzellbiokatalyse bekannt **[Ernst *et al.* 2005]**. Typische Vertreter sind die ADH aus Hefe (yeast alcohol dehydrogenase – Y-ADH) **[Miroliaei und Nemat-Gordani 2002]**, aus Pferdeleber (horse liver alcohol dehydrogenase (HL-ADH)) **[Muller *et al.* 2005]**) und verschiedenen Bakterienarten *(Rhodococcus ruber* – ADH-'A', *Thermoanaerobacter brockii* – TB-ADH und *Lactobacillus brevis* – LB-ADH) **[Olofsson *et al.* 2005]**. Zur Cofaktorregenerierung werden üblicherweise die substratgekoppelte oder

enzymgekoppelte Cofaktorregenerierung verwendet **[Daussmann 2005, Eckstein *et al.* 2004(a)]**.

Durch die große Vielzahl von verfügbaren Alkoholdehydrogenasen sind einige Substanzen bereits im multi-Kilogramm-Maßstab dargestellt worden **[Daussmann 2005, De Wildeman *et al.* 2007]** (Tabelle 4-2).

Tabelle 4-2: Industrielle Synthese von sekundären Alkoholen

Substanz	Hersteller
(*S*)-2-Butanol	IEP GMBH
Ethyl-4-(*S*)-chloro-3-hydroxybuttersäure	IEP GMBH
(*R*)-2-Octanol	Codexis

Darüber hinaus stellt die Leucin Dehydrogenase aus *Bacillus sphaericus* einen weiteren wichtigen Vertreter der Oxidoreduktasen dar. Unter Verwendung dieses Enzyms wurden bereits mehrere Tonnen *tert.*-Leucin dargestellt **[Liese *et al.* 2006]**. Eine Besonderheit ist, dass das Reaktionssystem inklusive Cofaktorregenerierung ebenso als Ganzzellbiokatalyse möglich ist **[Menzel *et al.* 2004]**.

Alkoholdehydrogenase aus *Lactobacillus brevis*

Ein außerordentlich erfolgreicher Vertreter dieser Gruppe ist die Alkoholdehydrogenase aus *Lactobacillus brevis* (LB-ADH) **[Schlieben *et al.* 2005]**. Das Enzym ist ein Tetramer bestehend aus 4 Untereinheiten von je 251 Aminosäuren und einer Gesamtmasse von 106,4 kDa. Sie ist NADPH-abhängig, *R*-selektiv und weist ein großes Substratspektrum von verschiedenen Ketonen auf. Bevorzugt werden Ketone mit einer Methyl-Seitenkette am prochiralen Substrat, wobei mit deutlicher Abstufung auch weitere Substrate (beispielsweise Ethylketone) umgesetzt werden. Wichtig sind hingegen 2 Zink-Ionen, welche aber nicht direkt in den katalytischen Zyklus eingehen. Außerdem ist die Gegenwart von Mg^{2+}-Ionen relevant, da diese indirekt durch eine koordinative Stabilisierung (Bindung von 4 Wassermolekülen und mehreren Seitenketten des Proteins) die Reaktivität des aktiven Zentrums ermöglichen (Abbildung 4-5). Das Substrat Acetophenon befindet sich rechts oben in der Grafik, der Cofaktor ist nicht dargestellt.

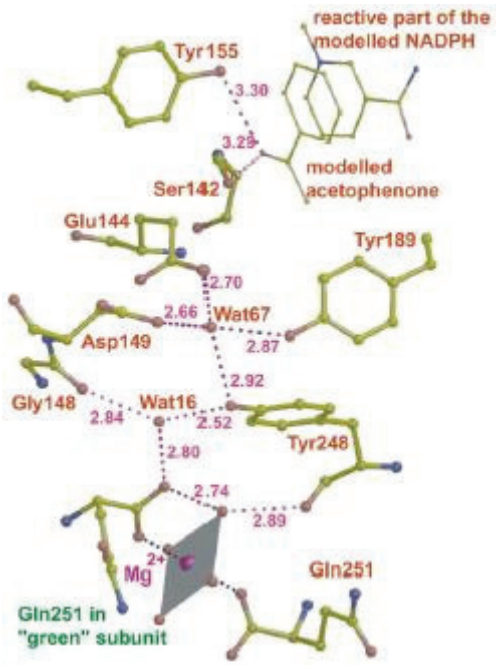


Abbildung 4-5: Mechanismus der Alkoholdehydrogenase aus *Lactobacillus brevis* **[Niefind *et al.* 2003]**

Die LB-ADH wurde bereits in verschiedenen nicht-wässrigen Reaktionssystemen eingesetzt: wässrig-organisches Zweiphasensystem; Wasser-Ionische Flüssigkeit-Zweiphasensystem **[Eckstein *et al.* 2004(b)]**; Verwendung in einer Gasphasenreaktion **[Ferloni *et al.* 2004]** und in immobilisierter Form in reinen organischen Lösungsmitteln **[Trivedi *et al.* 2005]**. Insbesondere die Verwendung in wässrig-organischen Zweiphasensystemen ist für die industrielle Synthese von verschiedenen chiralen Alkoholen von großer Bedeutung **[Daußmann *et al.* 2006]**.

5 Hydroxynitril Lyasen

5.1 Einleitung Cyanhydrine

Cyanhydrine stellen für die präparative Chemie eine interessante Gruppe von chemischen Zwischenprodukten dar, da sie in einem Molekül gleichzeitig zwei funktionelle Gruppen vereinen, die in Folgereaktionen noch weiter derivatisiert werden können **[Gregory 1999, North 2003]**. Die Synthese von Cyanhydrinen erfolgt in der Regel über die Addition von Blausäure an Aldehyde bzw. Ketone, wobei gleichzeitig eine Kettenverlängerung um eine C1-Einheit eintritt (Abbildung 5-1). Von synthetischer Relevanz ist darüber hinaus ebenso die direkte Synthese von trimethylsilylierten Cyanhydrinen. Das ungeschützte Cyanhydrin ist dann durch einen einfachen Hydrolyseschritt zugänglich. Erwähnenswert ist an dieser Stelle, dass sich die thermodynamischen Gleichgewichtszustände von beiden Reaktionen deutlich unterscheiden und Vergleiche zwischen diesen beiden Varianten nicht möglich sind. Unkatalysiert entsteht zusätzlich bei beiden Ansätzen ein racemisches Produkt.

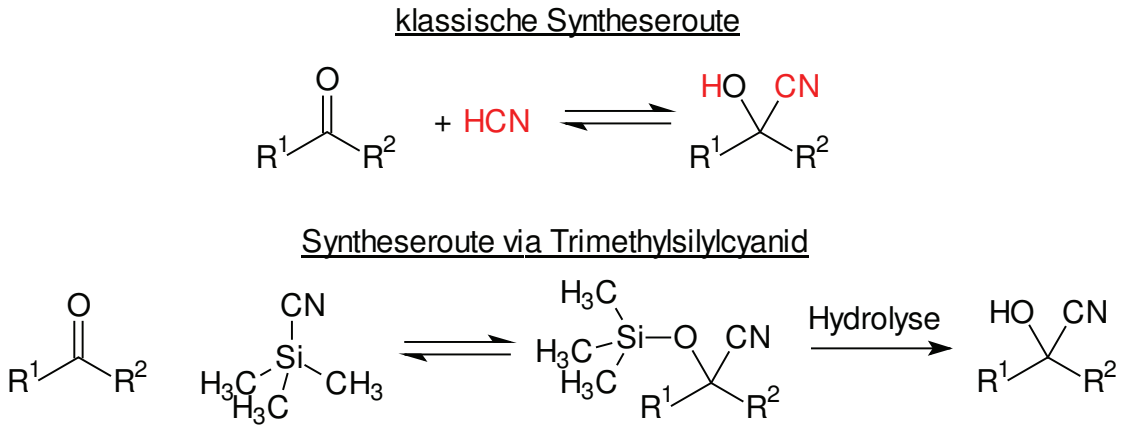


Abbildung 5-1: Cyanhydrinsyntheserouten

Die Besonderheit der Cyanhydrine liegt in dem ernormen Produktspektrum, welches durch eine große Folgechemie zugänglich ist. Die Möglichkeiten erstrecken sich über einfache Hydrolysereaktionen bis hin zu komplexen Cyclisierungsreaktionen (Abbildung 5-2) **[Bühler *et al.* 2000, Rosen und Daußmann 2005]**.

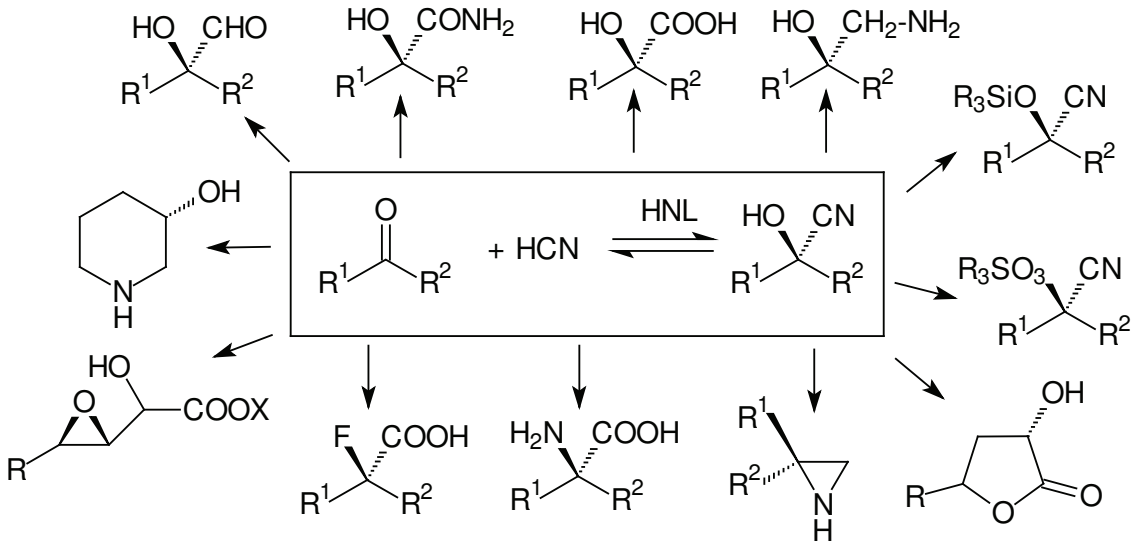


Abbildung 5-2: Mögliche Folgeprodukte aus enantiomerenreinen Cyanhydrinen

Besonders hervorzuheben ist an dieser Stelle der Bedarf an enantiomerenreinen Cyanhydrinen, welche vorrangig als Ausgangsstoffe für Feinchemikalien (z. Bsp. pharmazeutische Produkte) dienen **[Breuer *et al.* 2004, Daußmann *et al.* 2006, Knollmuller *et al.* 1999]**. Zur Gewinnung von enantiomerenreinen Cyanhydrinen eignen sich prinzipiell nur wenige Verfahren (Abbildung 5-3). Der direkte Weg der asymmetrischen Katalyse ermöglicht die Darstellung von Cyanhydrinen (bzw. trimethylsilylierte Cyanhydrine) ausgehend von den entsprechenden Aldehyden bzw. Ketonen. Neben den klassischen enzymatischen Verfahren sind auch metall- und organokatalytische Verfahren entwickelt worden.

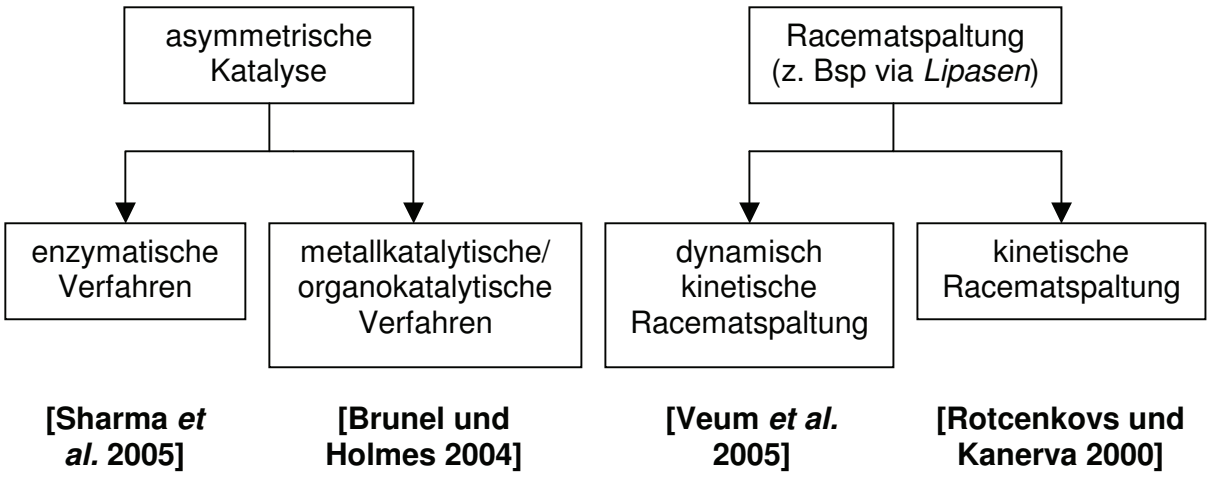


Abbildung 5-3: Verfahren zur Darstellung enantiomerenreiner Cyanhydrine

Eine alternative Route zur Darstellung enantiomerenreiner Cyanhydrine ist die (dynamisch) kinetische Racematspaltung mit Lipasen. Dies erfordert hingegen die

Verfügbarkeit des racemischen Cyanhydrins bzw. die entsprechende *in situ*-Darstellung und Racemisierung. Für die Racematspaltung sind nur enzymatische Verfahren bekannt, wobei der Einsatz von Lipasen einen überwiegenden Teil einnimmt.

5.2 Enzymatische Cyanhydrinsynthese

Hydroxynitril Lyasen (HNL's, auch: α-Hydroxynitril Lyasen bzw. Oxynitrilasen) gehören zur Gruppe der Lyasen (E.C. 4) und katalysieren die reversible Spaltung von Cyanhydrinen zu Aldehyden bzw. Ketonen, dabei wird Blausäure frei (Abbildung 5-4) **[Effenberger *et al.* 2000]**. Die Synthesereaktion erfolgt in umgekehrter Richtung und ermöglicht den Aufbau von Cyanhydrinen **[Becker *et al.* 1965, Griengl *et al.* 1997]**.

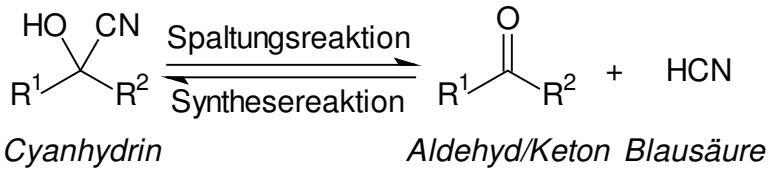


Abbildung 5-4: Cyanhydrinsynthese und -spaltung

Die natürliche Funktion der Hydroxynitril Lyasen liegt in ihrer Eigenschaft als Verteidigungsmechanismus. In zwei Schritten wird aus einem cyanogenen Glycosid das besonders für Warmblüter stark toxische Gift ‚Blausäure' freigesetzt, welches damit Fraßfeinde entgegenwirken soll **[van der Werf *et al.* 1994, Wolfsie und Shaffer 1959]**.

In der Nutzpflanze Maniok (*Manihot esculenta*) wird zum Beispiel das cyanogene Glycosid ‚Linamarin' in einem ersten Reaktionsschritt mittels einer Linamarinase in Glucose und Acetoncyanhydrin gespalten (Abbildung 5-5) **[Gruhnert *et al.* 1994]**. Im zweiten Reaktionsschritt katalysiert die Hydroxynitril Lyase aus *Manihot esculenta* (*Me*HNL) die Synthese von Aceton und Blausäure ausgehend von Acetoncyanhydrin. Das cyanogene Glycosid und die beiden Enzymsysteme sind bei einer unbeschädigten Pflanze räumlich getrennt (Kompartimentierung) und werden erst bei der Beschädigung der Zellwände zusammengeführt. Die Enzyme Linamarinase und Hydroxynitril Lyase sind im Falle des Manioks in der Zellwand und das Linamarin in den Vakuolen lokalisiert **[Mkpong *et al.* 1990]**.

Der Aufbau des Linamarin erfolgt in den Maniokblättern ausgehend vom L-Valin zum Oxim über die Cytochrom P450-Enzyme CYP79D1 und CYP79D2 **[Bak *et al.* 2000]**. Anschließend setzt ein weiteres Cytochrom P450-Enzym das Oxim zu Acetoncyanhydrin um. Dieses wird schließlich durch eine UDPG-Glucosyl-Transferase zum Linamarin umgesetzt und in der Zelle angereichert.

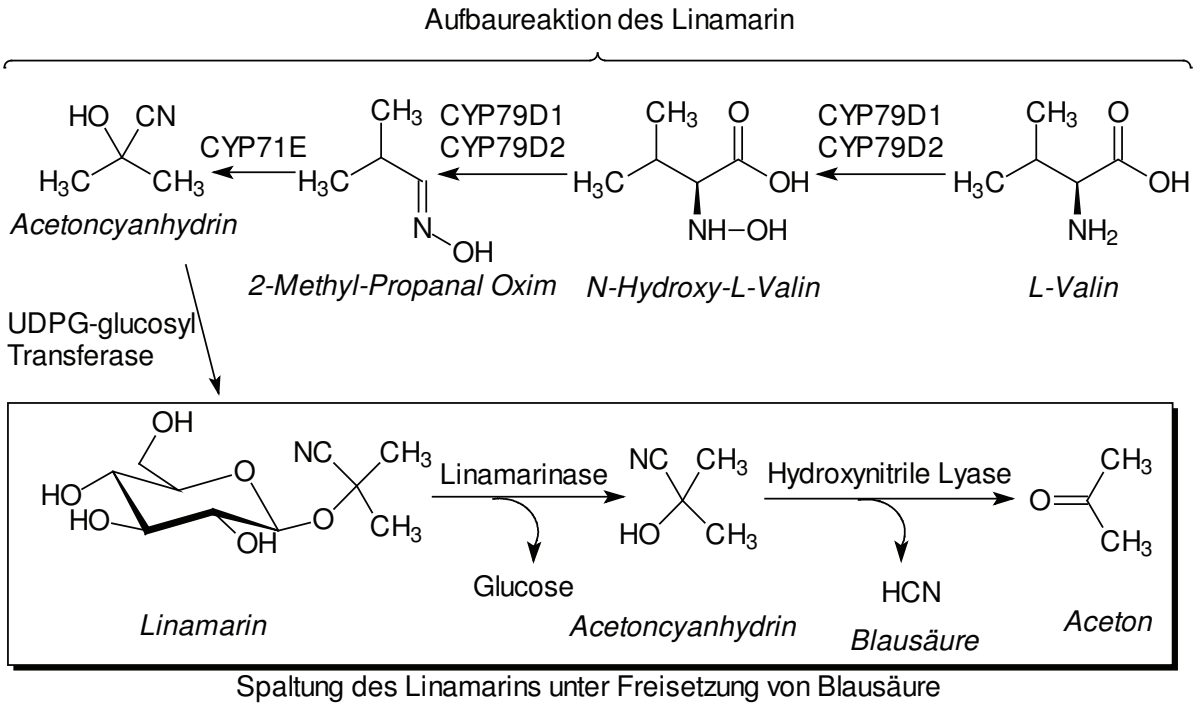


Abbildung 5-5: Biosynthese und Spaltung des Linamarins

Praxisrelevant ist hingegen die Synthesereaktion zu Cyanhydrinen, welche sehr einfach mit der Hydroxynitril Lyase bewerkstelligt werden kann. Diese Enzymreaktion stellt wahrscheinlich die erste asymmetrische Katalyse im Labor dar **[Rosenthaler 1908]**.

Die enzymatische Synthese der Cyanhydrine erfolgt ausgehend von Aldehyden bzw. Ketonen durch eine enantioselektive Addition der Blausäure. Interessanterweise weist eine Vielzahl von Hydroxynitril Lyasen eine solche Enantioselektivität auf, auch wenn das natürliche Substrat unter Umständen nicht chiral ist (Abbildung 5-6).

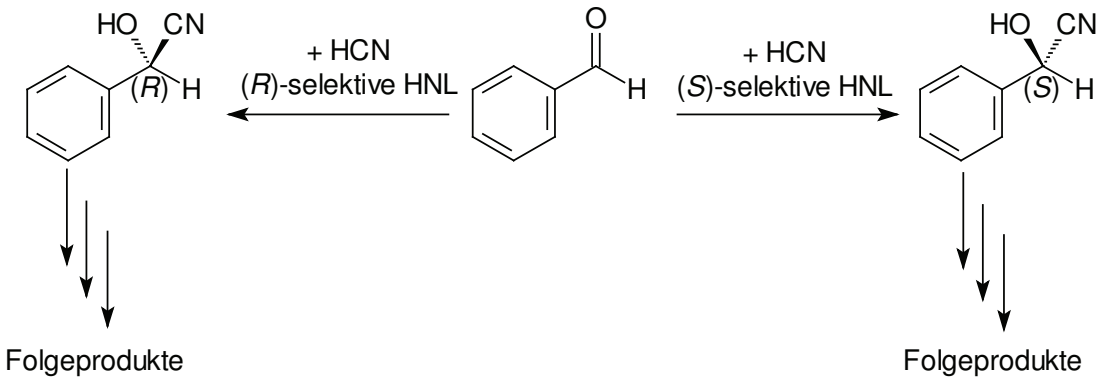


Abbildung 5-6: verschiedene Enantioselektivitäten am Beispiel des Benzaldehydcyanhydrin (Mandelonitril)

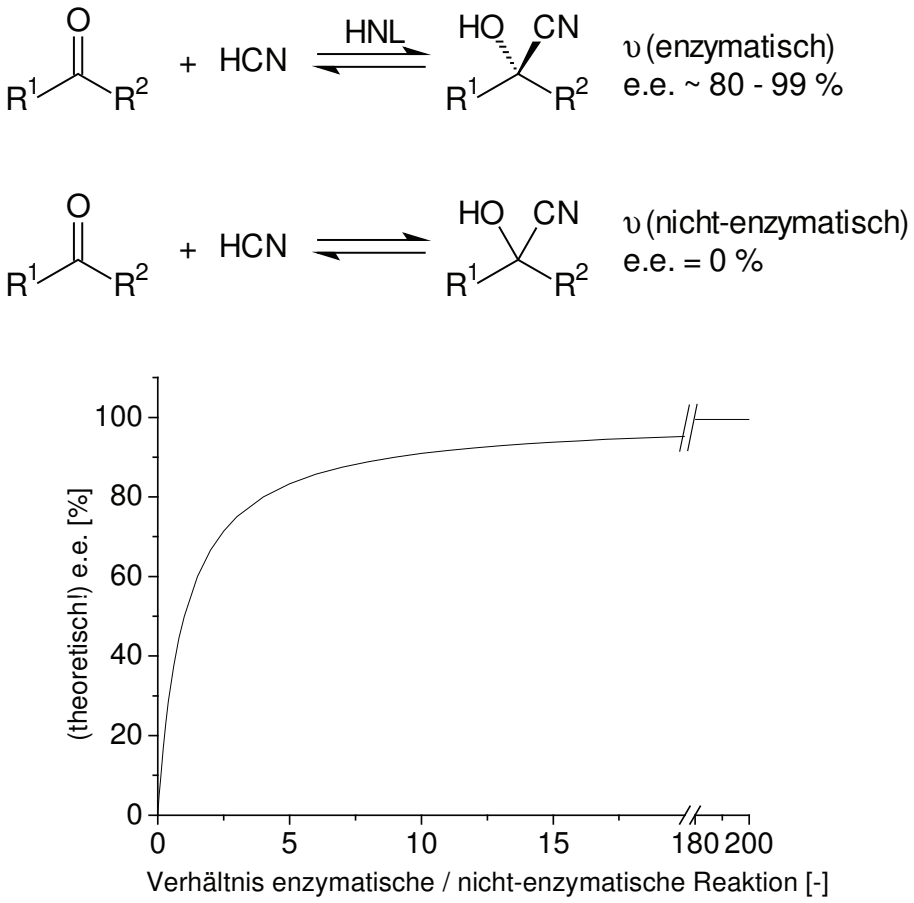


Abbildung 5-7: Enzymatische vs. nicht-enzymatische Reaktion

Das größte Problem der enzymatischen Cyanhydrinsynthese stellt dagegen die nicht-enzymatische Reaktion dar (Abbildung 5-7) (siehe **Publikation 4**). Während die enzymatische Reaktion üblicherweise hohe oder sehr hohe Enantiomerenüberschüsse erzeugt, wird durch die nicht-enzymatische (Neben-)Reaktion nur das racemische Produkt erhalten. Das Verhältnis der beiden parallel ablaufenden Reaktionen ergibt dabei den tatsächlichen verfügbaren Enantiomerenüberschuss des Produktes. υ

$$\frac{\upsilon(\text{enzymatisch})}{\upsilon(\text{nicht}-\text{enzymatisch})} \rightarrow \text{Enantioselektivität}$$

So ist beispielsweise für einen e.e.-Wert von 99 % ein Verhältnis der beiden Reaktionen (bzw. Reaktionsgeschwindigkeiten) von 100:1 und für 99.9 % von 1000:1 notwendig. Dementsprechend muss das Verhältnis deutlich zugunsten der enzymatischen Reaktion verschoben werden, was üblicherweise durch eine Unterdrückung der nicht-enzymatischen Reaktion erfolgt.

Klassischerweise wird dies über eine Verringerung der Temperatur und des pH-Wertes erreicht (Abbildung 5-8) (siehe auch **Publikation 4**). An die Verringerung des pH-Wertes ist aber, wie ebenfalls in Abbildung 5-8 dargestellt, eine Verringerung der Enzymaktivität gekoppelt. Zusätzlich sinkt die Prozessstabilität der verwendeten HNL durch diese Maßnahmen ebenfalls ab, was zusätzliche Enzymkosten verursacht. Ebenso sind Substrat- und Produktinhibierungen zu beachten, welche das Verhältnis der Reaktionsgeschwindigkeiten beeinflussen können.

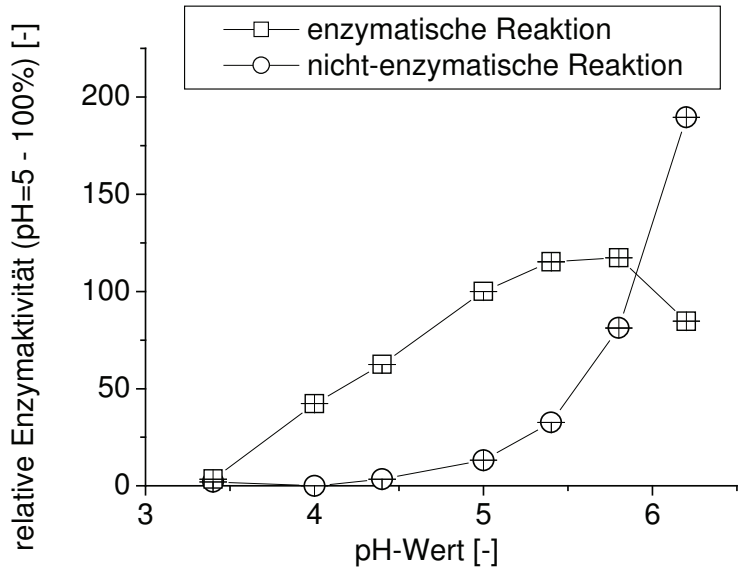


Abbildung 5-8: Unterdrückung der nicht-enzymatischen Cyanhydrinsynthese durch die Absenkung des pH-Wertes (pH = 5 entspricht 100%)

Nichtsdestotrotz stellt die HNL-katalysierte Cyanhydrinsynthese trotz diese Widrigkeiten durch gute Verfügbarkeiten der beteiligten Enzyme und ausgezeichnete Enantioselektivitäten einen hervorragende Route zu enantiomerenreinen Cyanhydrinen dar.

5.3 Stand der Technik

Seit den 1990er Jahren hat die enzymatische Cyanhydrinsynthese (via Hydroxynitril Lyasen) alle weiteren enantioselektiven Verfahren in der Cyanhydrinsynthese nahezu komplett verdrängt. Dieser Fakt beruht auf mehreren Ursachen **[North 1993]**.

1. Die milden Reaktionsbedingungen in der enzymatischen Cyanhydrinsynthese sind ein deutlicher Vorteil gegenüber den metall- bzw. organokatalytischen Syntheserouten. Zum einen wird in der enzymatischen Synthese oftmals Wasser als Reaktionsmedium verwendet und bei moderaten Temperaturen (5 °C bis

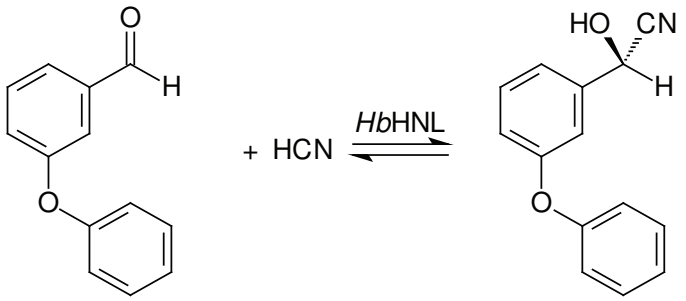
Raumtemperatur) die Reaktion durchgeführt. Metallkatalysierte Reaktionen benötigen dagegen wasser- und sauerstofffreie Bedingungen, bedingt durch die Verwendung von Trimethylsilylcyanid, und verlangen geringe Temperaturen (-20 bis -78 °C). In einigen Fällen sind auch hohe Drücke notwendig **[Choi *et al.* 1997]**.

2. Des Weiteren sind in den letzten beiden Jahrzehnten zahlreiche (*R*)- als auch (*S*)-selektive Hydroxynitril Lyasen isoliert worden, die ein enormes Substratspektrum beinhalten. Durch die moderne Molekularbiologie sind diese Enzyme ebenso als rekombinante Proteine (z. Bsp. via *Escherichia coli*) in großen Mengen bei geringen Kosten verfügbar.
3. Bedingt durch die thermische Instabilität der Cyanhydrine sind nur wenige racemische Cyanhydrine kommerziell verfügbar. Somit stellt die kinetische Racematspaltung mittels Hydroxynitril Lyasen (in der Spaltungsreaktion) zur Zeit nur eine untergeordnete Syntheseroute dar.
4. Dynamisch kinetische Racematspaltungen sind dagegen über Lipasen realisiert worden, welche die enantioselektive Acylierung von Cyanhydrinen katalysieren. Ungünstigerweise sind Reaktionszeiten von bis 5 Tagen notwendig, womit diese Variante ebenso wenig praktikabel ist.

In der Literatur sind zahlreiche HNL's beschrieben worden, aber zur Zeit werden nur folgende Hydroxynitril Lyasen intensiv untersucht bzw. wurden im industriellen Maßstab eingesetzt **[Poechlauer *et al.* 2004]**[1]. Zur Zeit stellt die Herstellung der Pestizidvorstufe (*S*)-3-Phenoxy-benzaldehydcyanhydrin mittels der Hydroxynitril Lyase aus *Hevea brasiliensis* (rekombinant aus *P. pastoris*, Firma DSM, Niederlande) die wichtigste großtechnische Anwendung dar (Abbildung 5-9).

Selektivität	Quelle
R-selektive HNL	*P. amygdalus* (bittere Mandel), *L. usitattissimum* (Flachs)
S-selektive HNL	*M. esculenta* (Maniok), *H. brasiliensis* (Kautschukbaum), *S. bicolor* (Hirse)

[1] Ergebnis aus einer Literaturrecherche der wissenschaftlichen Publikationen für die letzten 3 Jahre (Datenbank: Scifinder Scholar)



3-Phenoxybenzaldehyd (*S*)-3-Phenoxybenzaldehydcyanhydrin

Abbildung 5-9: Aktuell relevante Hydroxynitril Lyasen

Hydroxynitril Lyase aus *Manihot esculenta*

Ein wichtiger Vertreter innerhalb dieser Enzymgruppe ist die Hydroxynitril Lyase aus *Manihot esculenta* (*Me*HNL) (EC 4.1.2.37). Ein Enzymmolekül besteht aus 258 Aminosäuren mit einer Masse von 28.5 kDa und gehört in die Gruppe der α/β-Hydrolasen **[Ollis *et al.* 1992]**. In den katalytischen Zyklus gehen eine Reihe von Aminosäuren ein (Abbildung 5-10), wobei schlussendlich eine (*S*)-Selektivität auftritt.

Die *Me*HNL setzt sowohl Aldehyde als auch Ketone mit hohen Reaktivitäten und Enantioselektivitäten um (Tabelle 5-1) (siehe auch **[Bühler 2000, Wajant *et al.* 1996]**). Eine Besonderheit stellt das Substrat 3-Phenoxybenzaldehyd dar, welches durch seine sterische Beanspruchung bei den Standardreaktionsbedingungen (pH = 4–4.5) nur mit geringen Reaktionsgeschwindigkeiten umgesetzt wird. Die besonders geringe nicht-enzymatischen Reaktion ermöglicht an dieser Stelle aber auch deutlich höhere pH-Werte in der enzymatischen Reaktion, was zu sehr guten Produktivitäten führt (siehe auch **Publikation 4**). Darüber hinaus wurde gezeigt, dass der Austausch einer Aminosäure (Aufweitung des aktiven Zentrums) auch die Reaktionsgeschwindigkeit des Enzyms für sterisch anspruchsvolle Substrate deutlich anheben kann **[Lauble *et al.* 2002]**.

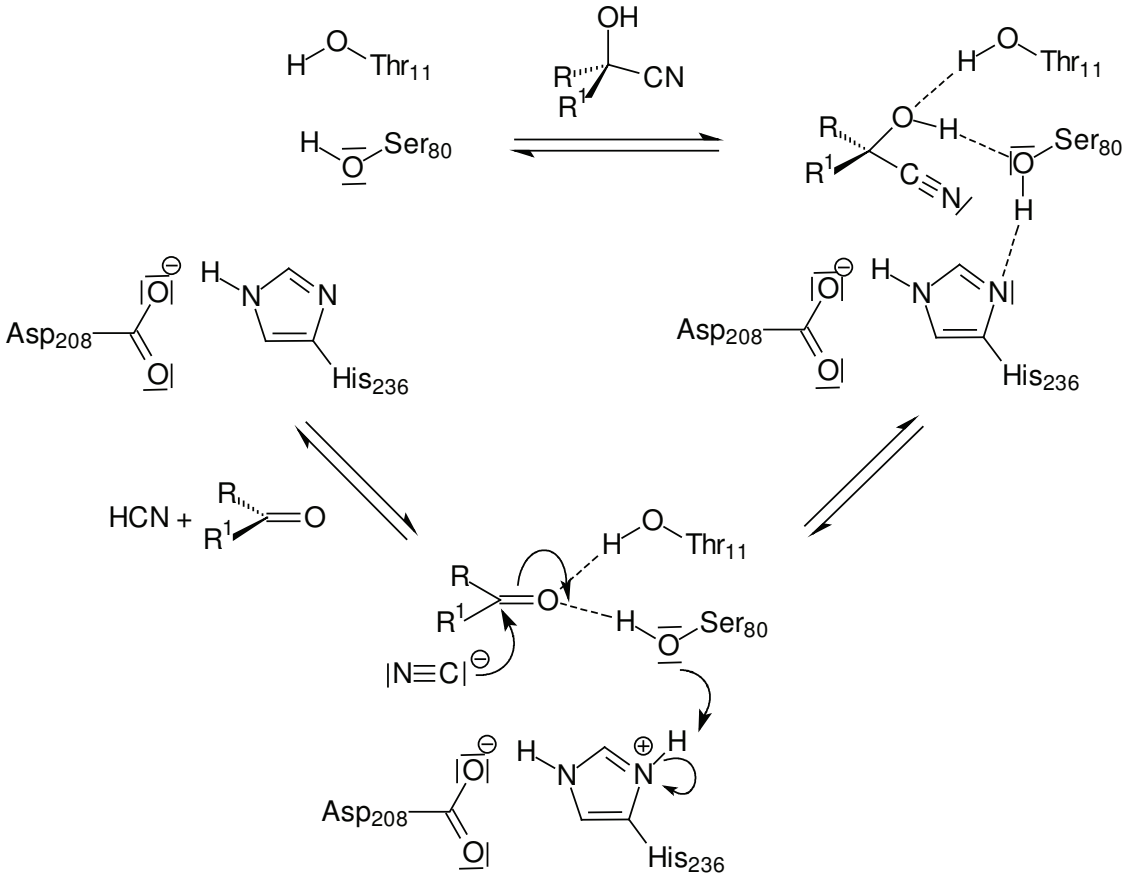


Abbildung 5-10: Mechanismus der katalytischen Cyanhydrin Synthese bzw. Spaltung der *Me*HNL **[Lauble *et al.* 2001]**

Durch das große Substratspektrum von aromatischen und aliphatischen Substraten und die hohe (*S*)-Enantioselektivität ist die *Me*HNL ein ausgezeichneter Katalysator in der enzymatischen Cyanhydrinsynthese (Tabelle 5-1).

Die *Me*HNL ist als rekombinantes Protein in großen Mengen verfügbar und weist zusätzlich eine ausgezeichnete Stabilität unter Prozessbedingungen auf (Halbwertszeit 8.7 d bei 10 °C im Zweiphasensystem Diisopropylether-Puffer). Durch die Kombination dieser positiven Eigenschaften ist die *Me*HNL bereits zur Darstellung mehrerer Tonnen (*S*)-2-Chlor-Mandelsäure (via (*S*)-2-Chlor-Mandelonitril) eingesetzt worden **[Wisdom 2007]**.

Tabelle 5-1: Substratspektrum der *Me*HNL

Substrat	Umsatz / %	e.e. (*S*) / %
Benzaldehyd	> 99	> 99
Phenylacetaldehyd	> 99	92
Hydrozimtaldehyd	> 99	69
3-Phenoxybenzaldehyd (pH=4)[1,2]	3	-
3-Phenoxybenzaldehyd (pH=6)[1,2]	90	95
Acetophenon[3]	2	-
Hexanal	> 99	86
Heptanal	98	80
Octanal	83	79
Nonanal	15	76
Decanal	15	75
2-Hexanon	95	58
2-Heptanon	n.b.	n.b.
2-Octanon	63	87
2-Nonanon	19	85
2-Decanon	10	87

Reaktionsbedingungen: 50 mmol/L Substrat, 250 mmol/L Blausäure, Zweiphasensystem: Diisopropylether – Citratpuffer pH=4, Reaktionszeit: 24h; n.b. – nicht bestimmt; [1] 22 h; [2] siehe auch **Publikation 4**; [3] siehe auch **Publikation 2**

6 Zusammenfassung

In der vorliegenden Arbeit wurden folgende Problemstellungen untersucht und Resultate erarbeitet:

1. Substratinhibierung

Anhand einer Alkoholdehydrogenase-katalysierten Reaktion wurde der systematische Einsatz von wassermischbaren organischen Lösungsmitteln untersucht. Durch die Verwendung von Acetonitril und 1,4-Dioxan ließ sich die Substratinhibierung von 2-Butanon bei der Alkoholdehydrogenase aus *Lactobacillus brevis* komplett unterdrücken. Somit wurde die **Produktivität verdreifacht** und darüber hinaus leicht die **Enantioselektivität erhöht**.

2. Ungünstige Gleichgewichtslage

Die enzymatische Acetophenoncyanhydrinsynthese ist durch die ungünstige Gleichgewichtslage von nur 2.5% Umsatz im Zweiphasensystem außerordentlich stark limitiert. Durch die Verwendung von lösungsmittelfreien Reaktionssystemen konnte der Gleichgewichtsumsatz auf bis zu 36% erhöht werden, was einer **Erhöhung um mehr als 1000%** entspricht. Außerdem wurden mehrere Acetophenonderivate untersucht, wobei mit 2'-Fluor-Acetophenon ein **Umsatz von 71%** möglich war. Zusätzlich konnten 5 ml enantiomerenreines (*S*)-Acetophenoncyanhydrin gewonnen werden.

3. Substratscreening

Die Isolierung einer neuartigen (*R*)-selektiven Hydroxynitril Lyase aus *Arabidopsis thaliana* erforderte die Bestimmung des optimalen Reaktionssystems und einer Übersicht über das Substratspektrum. Durch die Verwendung eines **Zweiphasensystems Diisopropylether-Puffer** konnte die **hohe Enantioselektivität und Produktivität** der *At*HNL ermittelt werden.

4. Optimierung der Prozessparameter im Zweiphasensystem

Hydroxynitril Lyase-katalysierte Reaktionen werden prinzipiell von der nicht-enzymatischen Cyanhydrinsynthese überlagert, wobei hierbei das ungewünschte Racemat entsteht. Unter Verwendung eines Zweiphasensystems und optimierten Reaktionsbedingungen konnten erstmals **hoch-pH-Reaktionsbedingungen** realisiert werden. Dabei konnte die Enzymstabilität und –aktivität positiv beeinflusst werden. Es war ebenfalls möglich das Substratspektrum des Wildtyp-Enzyms zu erweitern und **2 ml enantiomerenreines (*S*)-3-Phenoxybenzaldehydcyanhydrin** zu gewinnen.

5. Synthese von 5 x 50 g enantiomerenreiner Produkte

Ebenso wurden mittels der Hydroxynitril Lyase aus *Manihot esculenta* **5 x 50 g enantiomerenreine Cyanhydrine** dargestellt. Diese wurden nachfolgend zu den korrespondierenden α-Hydroxy-Carbonsäuren umgesetzt. **Raum-Zeit-Ausbeuten von ca. 12.5 g/(l·h)** wurden bestimmt, was das synthetische Potential von Hydroxynitril Lyasen deutlich hervorhebt.

Schlussfolgerungen

Durch die systematische Untersuchung konnten verallgemeinerte Aussagen zu enzymatischen Reaktionen in wässrig-organischen Reaktionsmedien erarbeitet werden.

- Unabhängig vom verwendeten Reaktionssystem zeigte sich, dass für die Produktgewinnung das wässrig-organische Zweiphasensystem im allgemeinen deutlich besser geeignet ist. Dieser Fakt gilt insbesondere für Reaktanden mit einer sehr geringen Wasserlöslichkeit. Die systematische Identifizierung der spezifischen Limitierungen ermöglichte in 3 verschiedenen Zweiphasensystemen die Produktisolierung im Gramm-Maßstab.
- Auf der anderen Seite sind für Reaktanden mit einer hohen Wasserlöslichkeit (siehe **Publikation 1 & 2**) Konzepte mit wassermischbaren organischen Lösungsmitteln eine mögliche Option, da Zweiphasensysteme hier kaum einen produktiven Nutzen haben. Somit beschränkt sich der Einsatzzweck auf sekundäre Einflüsse, wie zum Beispiel die Beeinflussung von kinetischen Parametern. Im vorliegenden Fall einer Alkoholdehydrogenase katalysierten Reaktion konnte ebenso gezeigt werden, dass bestimmte organische Lösungsmittel auch kinetische Konstanten beeinflussen können (siehe **Publikation 1**).

  Zusätzlich sind nur Lösungsmittel mit einem logP-Wert < 0 von Nutzen, da höhere Hydrophobizitäten (bis logP-Werte von 2 bis 4) meist eine Enzymdesaktivierung nach sich ziehen. Somit ist die Gruppe der möglichen Lösungsmittel sehr klein.
- Für einzelne Fälle (beispielsweise Acetophenon) bieten sich auch Grenzfälle der wässrig-organischen Reaktionssysteme an. So kann der löslichkeitsvermittelnde Effekt des organischen Lösungsmittels (=Substratphase) mit der Extraktion des Zweiphasensystems gekoppelt und damit eine Erhöhung des Gleichgewichtumsatzes ermöglicht werden (siehe **Publikation 2**).

Nichtsdestotrotz sind Abwandlungen von diesen Aussagen möglich, da immer mit weiteren enzymspezifischen Wechselwirkungen gerechnet werden muss. Ebenso sind für Ganzzellbiokatalysen, welche hierbei nicht beachtet wurden, weitere Effekte zu erwarten.

Abschließend formuliert stellen die organischen Lösungsmitteln auf Grund ihrer oben dargestellten Vorteile eines der wichtigsten Werkzeuge des ’Medium Engineering’ dar und werden auch zukünftig intensiv in der Biokatalyse verwendet werden (siehe Publikation 5).

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8 Publikationsanhang zur kumulativen Dissertation

1. Publikation 1

"Influence of water-miscible organic solvents on kinetics and enantioselectivitiy of the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis*"
Schumacher, J.; Eckstein, M.; Kragl,U.,
Biotechnology Journal, 1, 574-581 (2006)

2. **Publikation 2**

"Hydroxynitrile lyase in organic solvent free systems to overcome thermodynamic limitations"
von Langermann, J.; Mell, A.; Paetzold, E.; Daußmann, T.; Kragl, U.,
Advanced Synthesis and Catalysis, 349, 1418-1424 (2007)

3. **Publikation 3**

"A new (*R*)-selective Hydroxynitrile Lyase from Arabidopsis thaliana with an alpha/beta-Hydrolase fold"
Andexer, J.; **von Langermann, J.**; Mell, A.; Bocola, M.; Kragl, U.; Eggert, T.; Pohl, M.,
Angewandte Chemie - International Edition, 46, 8679-8681 (2007)

4. **Publikation 4**

"Hydroxynitrile lyase catalyzed cyanohydrin synthesis at high pH-values"
von Langermann, J.; Guterl, J.-K.; Pohl, M.; Wajant, H.; Kragl, U.,
Bioprocess and Biosystems Engineering, 31, 155-161 (2008)

5. **Publikation 5**

"Enzyme catalysis in non-aqueous media – past-present-future"
Dreyer, S.; Lembrecht, J.; **Schumacher, J.**, Kragl, U.
in R. Patel (Editor) "*Biocatalysis in the Pharmaceutical and Biotechnology Industries*" (2006), CRC-Press, Taylor & Francis Group, Boca Raton, pp 791-828

6. **Publikation 6**

"Hydroxynitrile lyase catalysed synthesis of enantiopure (*S*)-acetophenone cyanohydrins"
von Langermann, J.; Mell, A.; Paetzold, E.; Kragl, U.
in J. Whittal (Editor) "*Practical Methods in Biocatalysis and Biotransformations*", John Wiley & Sons Ltd, accepted

Publikation 1

“Influence of water-miscible organic solvents on kinetics and enantioselectivitiy of the (R)-specific alcohol dehydrogenase from Lactobacillus brevis”

Schumacher, J.; Eckstein, M.; Kragl,U.

Biotechnology Journal, 1, 574-581 (2006)

Anteile: **Schumacher J. (80%)**; Eckstein, M. (10%); Kragl, U. (10%)

Einleitung zu Publikation 1

Im Bereich der enzymatischen Synthese von enantiomerenreinen sekundären Alkoholen wurde für die Reduktion von 2-Butanon zu (*R*)-2-Butanol eine Substratinhibierung festgestellt.

Die übliche Vorgehensweise der Verwendung eines Zweiphasensystemes führt auf Grund der hohen Wasserlöslichkeit von 2-Butanon nicht zu einer nennenswerten Reduktion der Substratkonzentration in der wässrigen Phase. Dagegen ermöglicht die Zugabe von wassermischbaren organischen Lösungsmitteln eine deutliche Reduktion der Substratinhibierung bis zum Übergang zur klassischen Michaelis-Menten-Kinetik (Abbildung 8-1).

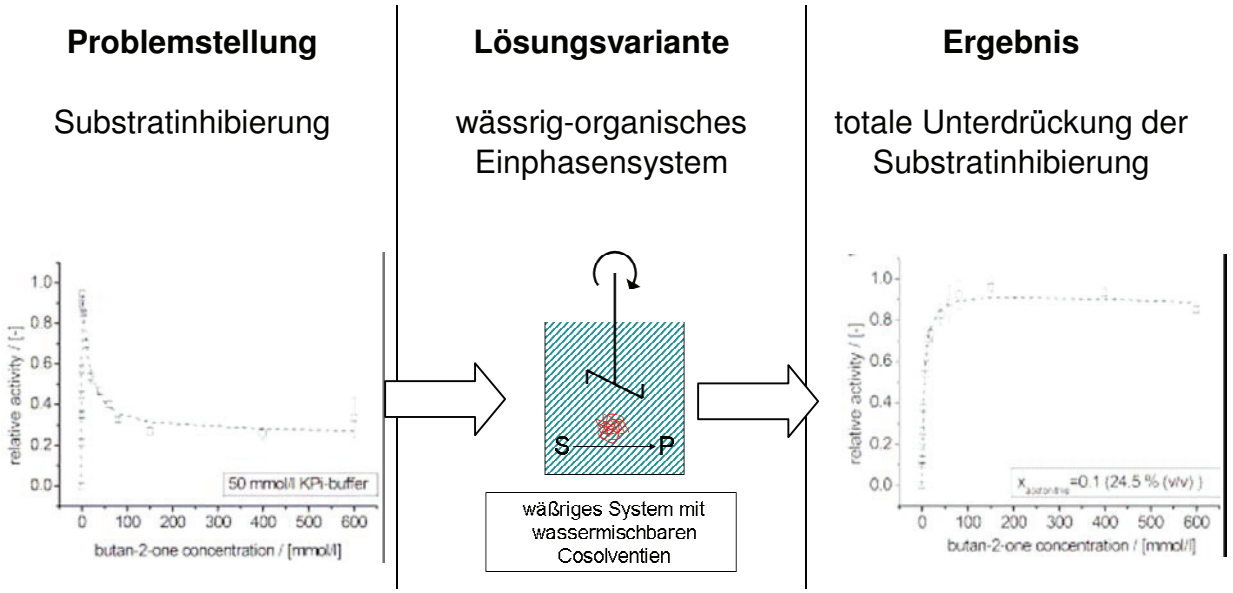

Abbildung 8-1: Vorgehensweise Publikation 1

Darüber hinaus wurde ermittelt, dass auf Grund der Tertiär-Struktur des Enzyms (Tetramer) eine besondere Wechselwirkung auftritt. Durch die Ausbildung eines Doppel-Substrat-Komplex (ESS) tritt eine Überlagerung der Substratinhibierung auf. Ein weiterer aktivierender Effekt, beschrieben durch die Effektorkonstante b, führt bei hohen Substratkonzentrationen zu einem Aktivitätsgewinn. Durch den Einsatz von 1,4-Dioxan und Acetonitril konnte somit durch die Produktivität und außerdem die Enantioselektivität leicht erhöht werden.

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Research Article

Influence of water-miscible organic solvents on kinetics and enantioselectivity of the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis*

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Using the organic solvents acetonitrile and 1,4-dioxane as water-miscible additives for the alcohol dehydrogenase (ADH)-catalyzed reduction of butan-2-one, we investigated the influence of the solvents on enzyme reaction behavior and enantioselectivity. The $NADP^+$-dependent (*R*)-selective ADH from *Lactobacillus brevis* (ADH-LB) was chosen as biocatalyst. For cofactor regeneration, the substrate-coupled approach using propan-2-ol as co-substrate was applied. Acetonitrile and 1,4-dioxane were tested from mole fraction 0.015 up to 0.1. Initial rate experiments revealed a complex kinetic behavior with enzyme activation caused by the substrate butan-2-one, and increasing K_M values with increasing solvent concentration. Furthermore, these experiments showed an enhancement of the enantioselectivity for (*R*)-butan-2-ol from 37% enantiomeric excess (ee) in pure phosphate buffer up to 43% ee in the presence of 0.1 mol fraction acetonitrile. Finally, the influence of the co-solvents on water activity of the reaction mixture and on enzyme stability was investigated.

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Keywords: Alcohol dehydrogenase · Organic solvent · One-phase system · Enantioselectivity · Kinetics

1 Introduction

Highly enantiopure secondary alcohols are important building blocks for various pharmaceuticals [1, 2]. In general, there are five possibilities to obtain these pure enantiomers: chemical catalysis, chiral pool approach, crystallization, chromatography and biocatalysis [3]. While chemical synthesis often suffers from limited enantioselectivity, biocatalysts are very selective catalysts for the production of numerous enantiopure products [4].

For the production of enantiopure secondary alcohols, alcohol dehydrogenases (ADH) from various sources showed an impressive synthetic potential [5, 6]. An interesting member of this group is the secondary ADH from *Lactobacillus brevis* (ADH-LB, EC 1.1.1.2), which exhibits high stereoselectivity and enantioselectivity for the reduction of a broad spectrum of prochiral ketones or keto acids as substrates [7]. However, ADHs require the reduced cofactor NAD(P)H that is converted in equimolar amounts compared to the substrate. Due to the high costs of NAD(P)H, a suitable cofactor regeneration is required [8]. Very common are the substrate-coupled cofactor regeneration, using propan-2-ol as co-substrate (see also Fig. 1) and the enzyme-coupled approach, using a second enzyme, *e.g.*, formate dehydrogenase and formate as co-substrate [9]. In addition, non-enzymatic regeneration pathways have been developed [10].

Conventional reaction media for biocatalytic processes are aqueous buffer solutions. However, several interesting substrates show extremely low solubilities in aqueous media. In this case, a co-solvent is needed to raise the substrate solubility in the reaction mixture. This co-solvent can either be water-miscible, resulting in a one-

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Abbreviations: ADH-LB, alcohol dehydrogenase from *Lactobacillus brevis*; **ee**, enantiomeric excess

phase system or it can be water-immiscible, leading to an aqueous-organic two-phase system [11]. The two phase system offers an easy work-up by phase separation and removal of the organic solvent. In addition, some other techniques have been developed, like enzyme immobilization [12] and encapsulation of the biocatalyst in reversed micelles [13], but they show only low usability for effective product recovery.

Nevertheless, the use of water-miscible co-solvents (one-phase system) is not limited to solubility effects [14]. Several enzymes also showed an enhancement of enzyme activity in the presence of water-miscible organic solvents [15]. Some authors have observed an influence on the enantioselectivity of secondary alcohol dehydrogenases from *Thermoanaerobacterium* SP. KET4B1 [16], *Thermoanaerobacter brockii* [17] and *Thermoanaerobacter ethanolicus* [18]. Furthermore, the addition of the water-miscible co-solvent acetonitrile was used to alter the cofactor specificity of a horse liver ADH (HL-ADH) [19]. Another interesting area for investigation is the interaction of organic solvents on kinetic parameters of enzyme reactions. A shift of the kinetic constants K_M and V_{max} can also yield in a higher productivity, as reported for several liver ADHs [20].

Here we describe the influence of two water-miscible organic solvents (acetonitrile and 1,4-dioxane) on the kinetic behavior and enantioselectivity of an ADH. Furthermore, the water activity and enzyme stability in presence of those co-solvents were of interest. Therefore, we chose to study the (*R*)-selective ADH from *Lactobacillus brevis*. This enzyme has shown an interesting resistance against water-miscible and immiscible organic solvents, which makes the ADH-LB an interesting enzyme even for large-scale applications [21]. Recent reports have also shown the usability in ionic liquids [22] and for gas-phase reactions [23].

As substrate, we chose butan-2-one to obtain (*R*)-butan-2-ol with high yields and high enantiomeric purities. (*R*)-Butan-2-ol is a useful building block for pharmaceuticals, flavors and perfumes [24].

2 Materials and methods

2.1 Chemicals and enzyme

NADPH and $NADP^+$ were purchased from Jülich Fine Chemicals (now: Julich Chiral Solutions) (Jülich, Germany). 2-Propanol, acetonitrile, dichloromethane, potassium dihydrogen phosphate and potassium hydrogen phosphate were obtained from J. T. Baker (Deventer, The Netherlands). 1,4-Dioxane was purchased from Riedel-de Haën (Seelze, Germany). Acetophenone was obtained from Merck (Hohenbrunn, Germany). Magnesium chloride and butan-2-one were of analytical grade.

The lyophilized ADH-LB (LOT 0511-0307) was obtained from Julich Chiral Solutions.

2.2 Preparation of reaction media containing water-miscible organic solvents

To obtain a 50 mmol/L phosphate buffer with 1 mmol/L $MgCl_2$, pH 7.0, a tenth of a tenfold concentrated buffer (500 mmol/l phosphate buffer with 10 mmol/L $MgCl_2$, pH 7.0) was used. The calculated amount of the water-miscible organic solvent was added to the concentrated buffer (see eq. 1). Subsequently, the required concentrated solutions of cofactor, substrate, co-substrate and enzyme were added to obtain the desired concentrations (see also Section 2.3). Missing volume was filled up with distilled water, which resulted in no significant change in apparent pH.

Equation 1:

$$V_{organic\ solvent} = \frac{\dfrac{V_{total}}{\dfrac{M_{organic\ solvent}}{\rho_{organic\ solvent}} + \dfrac{\left(1 - x_{organic\ solvent}\right)\cdot M_{water}}{x_{organic\ solvent}\cdot \rho_{water}}} \cdot M_{water}}{\rho_{organic\ solvent}}$$

$$and\ V_{water} = V_{total} - V_{organic\ solvent}$$

M – molar mass, ρ – density, x – mole fraction

2.3 Enzyme assay

The enzyme activity was measured by monitoring the consumption of NADPH at 340 nm in a 50 mmol/L phosphate buffer with 1 mmol/L $MgCl_2$, pH 7.0 at 30°C.

One unit of enzyme activity was defined as the amount of enzyme that catalyzes the reduction of 1 µmol acetophenone per minute under assay conditions with an extinction coefficient of 5.713 µmol/(min·mL). A SPECORD 200 from Analytikjena (Jena, Germany) equipped with quartz cuvettes was used.

For better comparison, all samples (containing organic solvent and butan-2-one) were compared with the standard assay under identical conditions (reduction of acetophenone at 10 mmol/L).

Equation 2:

$$\text{relative activity} = \frac{\text{measured activity (sample)}}{\text{measured activity (standard assay with 10 mM acetophenone)}}$$

All measurements were at least repeated three times and the average and SD were calculated. Only small changes of the extinction coefficient (less than 5%) have been observed.

2.4 GC analysis for determination of conversion and enantiomeric excess

The reaction mixture was extracted with dichloromethane and diluted with the same solvent, as appropriate. The conversion of butan-2-one to butan-2-ol was determined by GC analysis on a DB-1701 30 × 0.25 column from Alltech, with a flame ionization detector. The carrier gas was helium. The temperature gradient was 35°C for 1 min, rising at 0.5°C/min to 40°C, holding at 40°C for 1 min, rising at 40°C/min to 250°C and holding for 2 min at 250°C. The injector and flame ionization detection (FID) temperature was set to 250°C.

To determine the enantiomeric excess (ee), the reaction mixture was extracted with chloroform, dried over sodium sulfate and analyzed by GC-FID on a chiral stationary phase using a CP-Chirasil-DEX CB 25 × 0.25. Helium was used as carrier gas. The accuracy for the GC measurements was ±5% or better.

2.5 Water activity

Water activities were measured using an AW Sprint (TH-500) by Novasina (Pfaffikon, Switzerland), equipped with an activated charcoal filter for detector protection at 30°C.

3 Results

3.1 Influence of CO-solvents

In biocatalytic reactions several limitations, like substrate and product inhibition, may occur, which directly lead to a limitation in productivity. One way to overcome the problem of inhibition is the right choice of reactor type; a substrate inhibition can be overcome, for example, by a fed-batch process or by performing the reaction in a continuously operated stirred tank reactor. In case of product inhibition, an *in situ* product removal would be the best choice. Usually aqueous/organic two-phase systems and XAD-resins (for adsorption of the product) are used for this approach [25].

An alternative attempt to minimize inhibitory effects and side products is the use of the so-called "medium engineering", *i.e.*, the reaction medium may be optimized by addition of co-solvents [26–28] or by variation of reaction temperature [29]. It is known that different additives like organic solvents (water-immiscible and miscible), salts (*e.g.*, ionic liquids) and other organic compounds influence enzyme kinetics.

In this study, we investigated the influence of two water-miscible organic solvents on the substrate inhibition of an (*R*)-specific secondary ADH-LB for the reduction of butan-2-one. Furthermore, we examined the usability of these solvents for overcoming substrate inhibition at high substrate concentrations in large scale applications (Fig. 1). In addition, the influence on the enzyme's enantioselectivity for the reduction was examined.

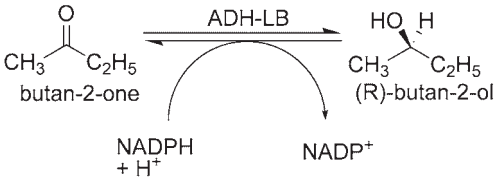


Figure 1. Reaction scheme for reduction of butan-2-one.

3.2 Initial rate experiments

Initial rate experiments revealed a complex kinetic behavior for ADH-LB in buffer and several mixtures of buffer and water-miscible organic solvent. Using pure phosphate buffer (KPi buffer) a strong substrate inhibition occurs, indicated by a decrease of the relative activity up to 30% after addition of 10–600 mmol/L butan-2-one. At first sight, a simple type of substrate inhibition appears to occur, although we have shown this not to be the case, as discussed below (Fig. 2).

Interestingly, the addition of acetonitrile causes a decrease in the magnitude of this substrate inhibition. At a mole fraction of 0.1 for acetonitrile (approximately a content of 24.5% acetonitrile in phosphate buffer), the substrate inhibition is hardly detectable (Fig. 3a–d).

In contrast to the addition of acetonitrile, the presence of 1,4-dioxane leads to a significant loss of the enzyme's maximum activity on increasing the amount of the organic solvent (Fig. 4a–d). Less than 50% of the maximum velocity obtained in pure phosphate buffer can be obtained at a mole fraction of 0.1 for 1,4-dioxane (approximately a content of 34.3% 1,4-dioxane in phosphate buffer). In comparison to the addition of acetonitrile, a reduction in substrate inhibition also appears, but only moderately.

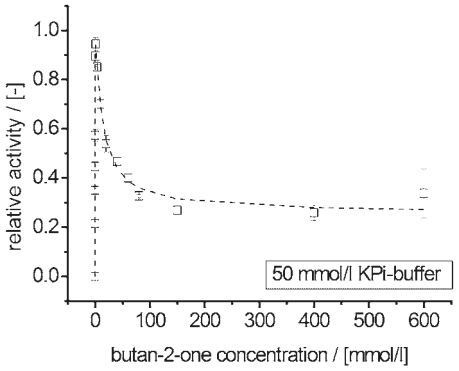


Figure 2. Initial rate experiments in 50 mmol/L KPi buffer pH 7.0; line: calculation using the kinetic model described below.

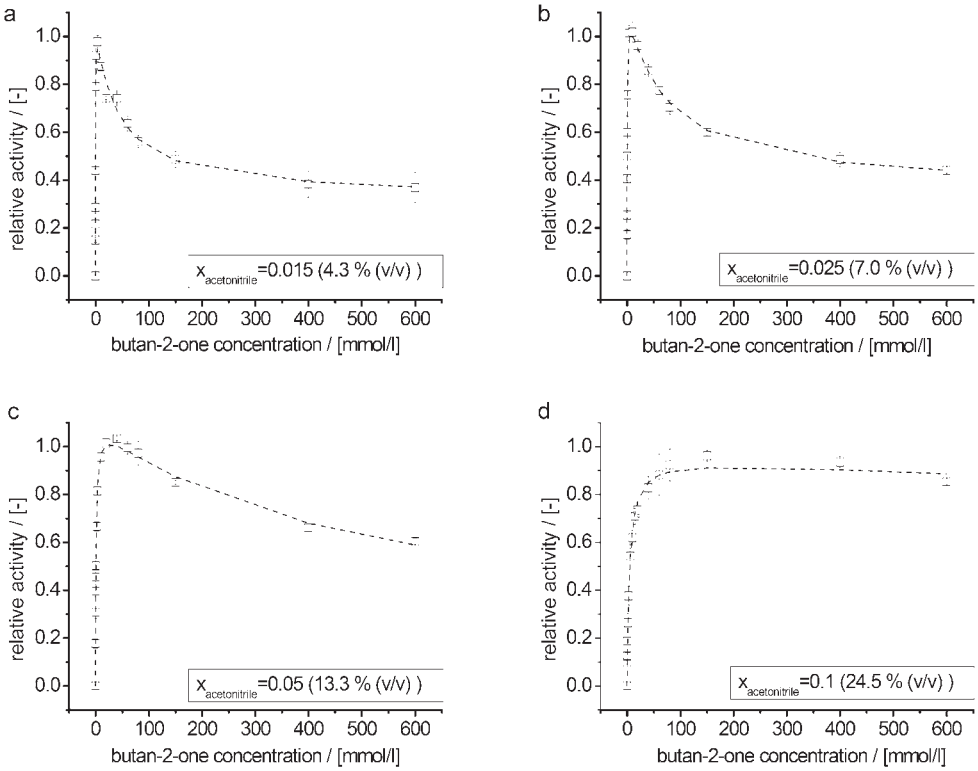


Figure 3. (a–d) Initial rate experiments in the presence of the co-solvent acetonitrile (expressed in molar fractions $x_{acetonitrile}$); line: calculation using the kinetic model described below.

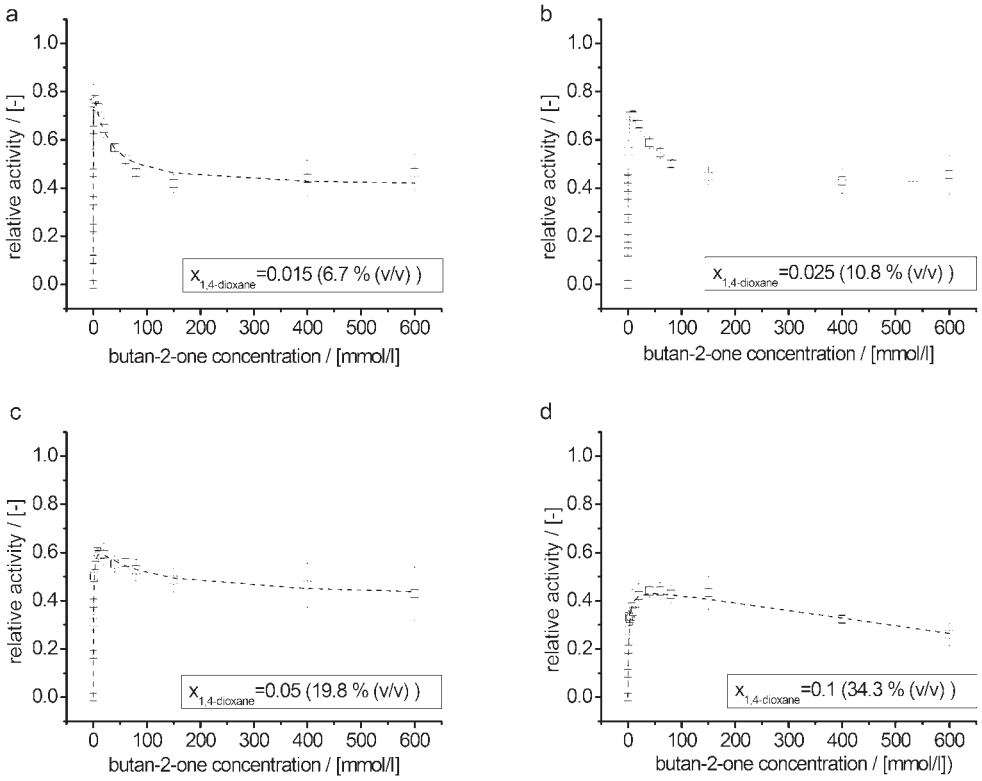


Figure 4. (a–d) Initial rate experiments in the presence of the co-solvent 1,4-dioxane (expressed in molar fractions $x_{1,4\text{-dioxane}}$), line: calculation using the kinetic model described below.

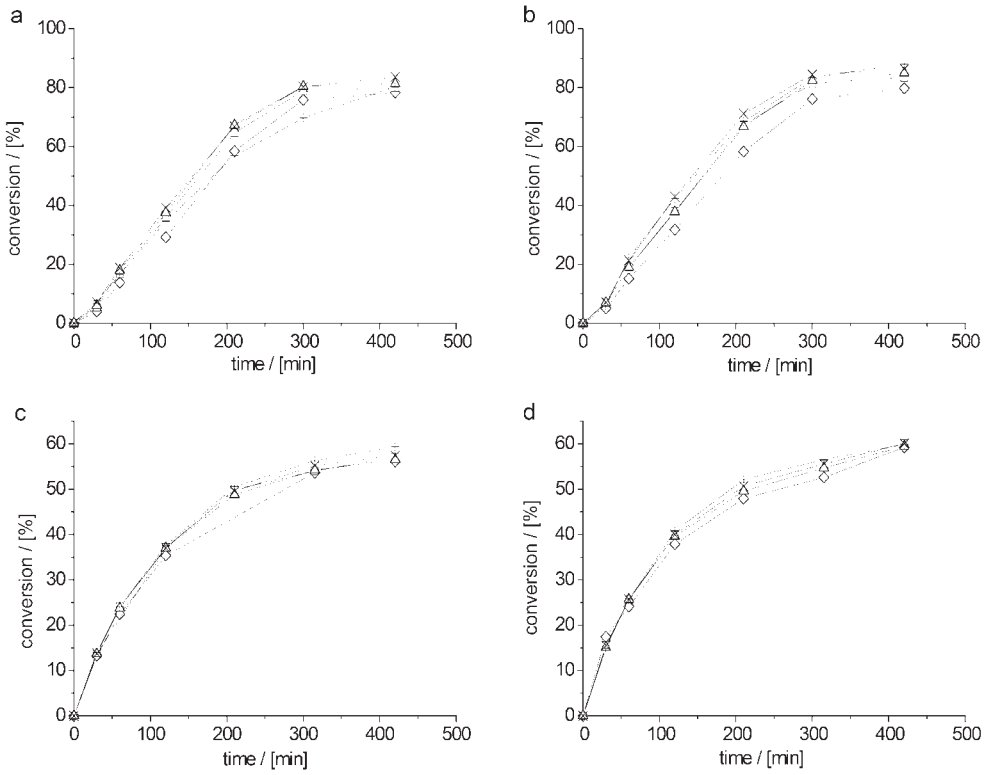


Figure 5. Conversion of the LB-ADH-catalyzed reduction of butan-2-one in the presence of acetonitrile and 1,4-dioxane, using 10 mmol/L and 600 mmol/L substrate (use of substrate-coupled cofactor regeneration with propan-2-ol as co-substrate). Enzyme activities were measured with the substrate acetophenone as described in Section 2. (◇) 50 mmol/L KPi buffer, (□) $x_{organic\ solvent} = 0.015$, (△) $x_{organic\ solvent} = 0.025$, (×) $x_{organic\ solvent} = 0.05$, (+) $x_{organic\ solvent} = 0.1$. (a) Addition of acetonitrile, 10 mmol/L butan-2-one, 200 mmol/L propan-2-ol, 0.1 mmol/L $NADP^+$, 30°C, 0.67 U/mL. (b) Addition of 1,4-dioxane, 10 mmol/L butan-2-one, 200 mmol/L propan-2-ol, 0.1 mmol/L $NADP^+$, 30°C, 0.67 U/mL. (c) Addition of acetonitrile, 600 mmol/L butan-2-one, 1200 mmol/L propan-2-ol, 6.0 mmol/L $NADP^+$, 30°C, 40.2 U/mL. (d) Addition of 1,4-dioxane, 600 mmol/L butan-2-one, 1200 mmol/L propan-2-ol, 6.0 mmol/L $NADP^+$, 30°C, 40.2 U/mL.

3.3 Enzyme stability and selectivity

For several enzymes (including oxidoreductases), it is known that water-miscible organic solvents have a negative effect on enzyme stability, whereas water-immiscible organic solvents like methyl-*tert.*-butyl-ether have been shown to stabilize the ADH-LB [30]. As anticipated, acetonitrile and 1,4-dioxane had an increasingly negative effect on the enzyme stability with increasing concentration of co-solvent added (Table 1). At this point two antipodal effects occur, an activating effect due to the decrease of substrate inhibition and a deactivating effect resulting from the reduction of enzyme stability. As shown in Fig. 5, at high and also low substrate concentrations, the influences on the ADH-LB perfectly overlap, yielding in similar reaction rates for all concentrations of added organic solvents. A slight acceleration was observed only for the reduction of 600 mmol/L butan-2-one using acetonitrile as co-solvent, and for 10 mmol/L butan-2-one in the presence of 1,4-dioxane.

The influence of acetonitrile and 1,4-dioxane on the enantioselectivity of the secondary ADH-LB was also in-

Table 1. Water activity (a_w), enzyme stability and ee for different solvent content[a)]

Reaction medium	a_w	Enzyme stability half life time (h)	ee (%)
50 mmol/L KPi buffer pH 7		**399**	**37**
$x_{acetonitrile} = 0.015$	0.956	159	33
$x_{acetonitrile} = 0.025$	0.955	121	41
$x_{acetonitrile} = 0.050$	0.943	34	43
$x_{acetonitrile} = 0.100$	0.936	1.6	43
$x_{1,4\text{-dioxane}} = 0.015$	0.944	49	34
$x_{1,4\text{-dioxane}} = 0.025$	0.94	45	32
$x_{1,4\text{-dioxane}} = 0.050$	0.889	43	34
$x_{1,4\text{-dioxane}} = 0.100$	0.832	26	40

a) Enzyme stabilities for LB-ADH in the different mixtures have been measured at 30°C as described in Section 2, using the enzyme assay with acetophenone. ee was investigated at 600 mmol/L butan-2-one, 1200 mmol/L propan-2-ol, 6.0 mmol/L $NADP^+$, 30°C, 40.2 U/mL (acetophenone) after reaching the reaction equilibrium.

vestigated. The data are compiled in Table 1. Besides a minimum in enantioselectivity at $x_{acetonitrile}$ = 0.015, a slight rise up to 43% ee (*R*) was observed in the presence of acetonitrile. For 1,4-dioxane the enantioselectivity decreased from 36% ee (*R*) (pure phosphate buffer) to 32.2% ee (*R*) ($x_{1,4\text{-dioxane}}$ = 0.025), and then increased in analogy to the addition of acetonitrile up to 40% ee (*R*). At high concentrations, the organic solvents potentially interact with the surface of the enzyme or with the active site itself, and alter the structure to a more rigid type, which can lead to higher enantioselectivities [31].

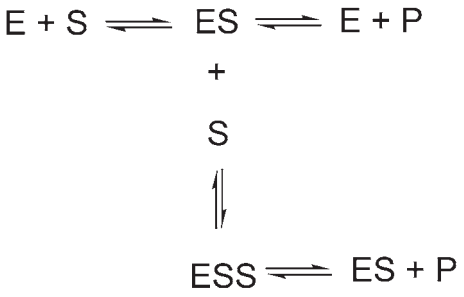
4 Discussion

We investigated the influence of the organic solvents acetonitrile and 1,4-dioxane on enzyme kinetics, enzyme stability and on the enantioselectivity of secondary ADH-LB.

The aim of the work was to find a kinetic model to explain the observed influence of the organic solvents on the suppression of substrate inhibition. The collected data are also used to discuss the reaction rate depending on the concentration of the organic co-solvent.

Usually, in initial rate experiments a simple substrate inhibition leads to a total inhibition of enzyme activity at high substrate concentrations. Dissecting the results for the initial rate experiments for the reduction of butan-2-one (shown in Figs. 3a–d, 4a–d) more precisely, an additional activating effect is seen to overlap the inhibitory effect, so that the total enzyme deactivation is avoided.

A more theoretical description of this behavior has already been described by Segel [32] and in a more universal way by Lasch [33]. Both authors postulate that the suppression of substrate inhibition may be a result of a second substrate (S) binding to the enzyme-substrate-complex (ES), building a double-substrate-enzyme complex (ESS). In contrast to simple substrate inhibition with an inactivated double-substrate-enzyme complex (ESS), the ESS formed in this case is more active than the enzyme-substrate complex (ES) itself (Fig. 6). This behavior may be explained by a conformational change of the enzyme caused by the addition of the chosen co-solvents [34].



$$\frac{v}{V_{max}} = \frac{S_0 + b \cdot \frac{S_0^2}{K_S'}}{K_S + S_0 + \frac{S_0^2}{K_S'}}$$

Figure 6. Kinetic model.

The formation of the double-substrate-enzyme complex (ESS) is described by the equilibrium constant K_S'. In addition to the enzyme activity, represented by k_{cat}, the ESS influences enzyme activity and is represented by the 'effector-constant' b. In case of an inactive ESS, the effector-constant b is set to zero and a simple substrate inhibition occurs. Values above zero (b >0) describe an activation that mostly appears at high substrate concentrations, and has also been observed in our experiments.

The results of the kinetic model, describing enzyme activity in the presence of acetonitrile as co-solvent were obtained by non-linear regression and are summarized in Table 2. Correlating V_{max} to the relative activity, V_{max} is non-dimensional in this case (see Section 2).

On the one hand, K_S rises from 0.09 mmol/L in pure buffer to 4.595 mmol/L in the presence of acetonitrile at $x_{acetonitrile}$ = 0.1. This is due to a change in the partitioning behavior of the substrate between the active site of the enzyme and the bulk phase caused by the co-solvent [35]. Working at fixed substrate concentrations, *e.g.*, 5 mmol/L,

Table 2. Kinetic parameters for the ADH-LB-catalyzed reduction of butan-2-one using co-solvents

Reaction medium	K_S / (mmol/L)	V_{max}	K_S' (mmol/L)	b
50 mmol/L KPi buffer pH 7	**0.09 ± 0.01**	**1.08 ± 0.03**	**11.20 ± 1.64**	**0.24 ± 0.02**
$x_{acetonitrile}$ = 0.015	0.15 ± 0.01	1.05 ± 0.03	41.22 ± 7.18	0.31 ± 0.02
$x_{acetonitrile}$ = 0.025	0.41 ± 0.03	1.14 ± 0.03	70.36 ± 12.49	0.32 ± 0.03
$x_{acetonitrile}$ = 0.050	1.17 ± 0.07	1.10 ± 0.02	464.74 ± 179.62	0.18 ± 0.15
$x_{acetonitrile}$ = 0.100	4.59 ± 0.48	0.96 ± 0.03	–	–
$x_{1,4\text{-dioxane}}$ = 0.015	0.10 ± 0.01	0.84 ± 0.03	23.53 ± 7.57	0.48 ± 0.03
$x_{1,4\text{-dioxane}}$ = 0.025	0.38 ± 0.04	0.80 ± 0.03	34.77 ± 9.79	0.51 ± 0.03
$x_{1,4\text{-dioxane}}$ = 0.050	0.62 ± 0.05	0.65 ± 0.01	81.77 ± 25.30	0.63 ± 0.03
$x_{1,4\text{-dioxane}}$ = 0.100	1.51 ± 0.18	0.46 ± 0.01	–	–

would simulate a remarkable loss of enzyme activity due to the shown significant change of K_S.

On the other hand, V_{max} remains nearly constant with a slight maximum for $x_{acetonitrile} = 0.025$. As discussed above, with a decrease of substrate inhibition, K_S' increases, resulting in a simple Michaelis-Menten kinetic at $x_{acetonitrile} = 0.1$. The effector constant b, which is only affected by the substrate butan-2-one, remains nearly constant around 0.3. As a consequence, the auxiliary solvent does not have any direct inhibitory influence on the active site of the enzyme. Consequently, V_{max} is not affected by the addition of acetonitrile.

For 1,4-dioxane a different behavior was found. As already discussed above, an additional loss of enzyme activity with increasing 1,4-dioxane concentration was observed (Table 2). Comparable to the results obtained for the ADH-LB-catalyzed reduction of butan-2-one in the presence of acetonitrile, a more moderate rise in K_S and K_S' was observed in the presence of 1,4-dioxane. In contrast to the results obtained in the presence of acetonitrile, V_{max} decreased to 0.458 at $x_{1,4\text{-dioxane}} = 0.1$, indicating a direct influence on enzyme conformation. Furthermore, an increase in the effector constant b was observed, which suggests further interactions between enzyme and the co-solvent 1,4-dioxane.

On the one hand, the organic co-solvent itself may have a direct influence on the enzyme conformation, leading to a loss of maximum velocity V_{max} in the presence of 1,4-dioxane [34]. However, this behavior is not easily associated with the physical properties of the added solvents. Several attempts to introduce the log-*P*-concept led to general rules for the usability of organic solvents in enzyme chemistry [30, 36, 37].

On the other hand, the change in conformation and thus the loss in enzyme activity may also be caused by a change in water activity. The addition of water-miscible organic solvents is known to lower the water activity in aqueous media [38]. In general, ADHs require high water activities for maximum activity [35]. Table 1 shows the measured water activities obtained for the investigated reaction mixtures using acetonitrile and 1,4-dioxane as co-solvents. For the addition of both solvents, only a small decrease in water activity with increasing concentration of co-solvent was observed. However, in the case of 1,4-dioxane the loss in water activity is slightly higher, leading to a water activity of about 0.84 in the presence of 1,4-dioxane with a molar fraction of 0.1. This could possibly explain the loss in maximum velocity (Table 1). Nevertheless, the usability of the water activity concept is still limited [38].

In conclusion, the ADH-LB was inhibited by the substrate butan-2-one. This substrate inhibition could be minimized by the addition of different amounts of the co-solvents acetonitrile and 1,4-dioxane. To find a suitable mathematical description of this behavior, a kinetic model was used, suggesting a double-substrate-enzyme complex (ESS) that is more active than the substrate-enzyme complex (ES). Thus, a substrate inhibition can be compensated by higher enzyme activities in the presence of co-solvents. A loss in maximum velocity V_{max} using 1,4-dioxane as co-solvent may be a consequence of lower water activities in the reaction mixture compared to the reaction conditions in the presence of acetonitrile.

Furthermore, the interaction of the chosen water-miscible organic solvents with the enzyme surface enhances the ee of the product (*R*)-butan-2-ol from 37% ee (*R*) in buffer to 43% ee (*R*) (acetonitrile) and 40% ee (*R*) (1,4-dioxane) (Table 1). These results show that water-miscible organic solvents can be a useful application for altering the ADH-LB-catalyzed enantioselective reduction of butan-2-one to (*R*)-butan-2-ol. Although the effect is low, there is a clear tendency to be seen from the GC chromatograms.

We thank Julich Chiral Solutions for the ADH from Lactobacillus brevis and determination of the ee. Part of this work was financially supported by the Ministry of Science and Education of Mecklenburg-Vorpommern within the HWP-program.

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Publikation 2

“Hydroxynitrile lyase in organic solvent free systems to overcome thermodynamic limitations”

von Langermann, J.; Mell, A.; Paetzold, E.; Daußmann, T.; Kragl, U.
Advanced Synthesis and Catalysis, 349, 1418-1424 (2007)

Anteile: **von Langermann (80%)**; Mell, A. (2,5%); Paetzold, E. (2,5%); Daußmann, T. (5%); Kragl, U. (10%)

Einleitung zu Publikation 2

Hydroxynitril Lyase katalysierte Reaktionen erbringen in vielen Fällen gute Umsätze und Enantioselektivitäten. Nichtsdestotrotz weisen insbesondere Ketonsubstrate meist nur ungenügend hohe Gleichgewichtsumsätze auf, was in der (synthetisch orientierten) Literatur mit einer geringeren ‚Stabilität' der Ketoncyanhydrine diskutiert wird. Interessanterweise finden sich aber durchaus viele Ketonsubstrate, welche durch Hydroxynitril Lyasen mit hohen Umsätzen und guten Enantioselektivitäten umgesetzt werden.

Aus diesem Grund wurde in der vorliegenden Publikation 2 an einem Modelsubstrat (Acetophenon) die Ursache der genannten Limitierung untersucht und Lösungsansätze diskutiert (Abbildung 8-2).

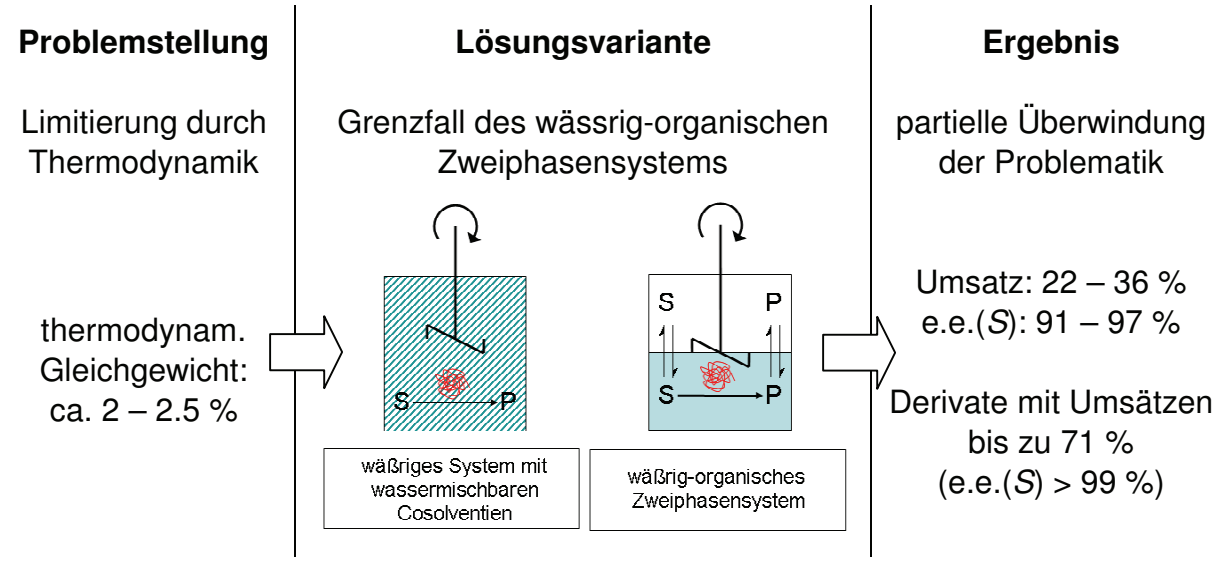

Abbildung 8-2: Vorgehensweise Publikation 2

Es zeigte sich, dass die thermodynamische Limitierung die geringen Gleichgewichtsumsätze auslöst. Substrat- und Produktinhibierungen konnten ausgeschlossen werden. Durch den Einsatz eines Grenzfalles des wässrig-organischen Zweiphasensystems (Substrat als organisches Lösungsmittel und Extraktionsmittel) konnte die Effektivität deutlich gesteigert und ein breites Substratspektrum untersucht werden. Abschließend konnten 5 ml enantiomerenreines (*S*)-Acetophenoncyanhydrin hergestellt werden.

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Hydroxynitrile Lyase in Organic Solvent-Free Systems to Overcome Thermodynamic Limitations

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Dedicated to Prof. Dr. Maria-Regina Kula on the occasion of her 70th birthday.

Abstract: The overcoming of thermodynamic limitations in the synthesis of optically active ketone cyanohydrins by using organic solvent-free systems has been investigated. Therefore, substrates with known unfavorable results within hydroxynitrile lyase-catalyzed reactions were selected for the determination of limitations and bottlenecks in ketone cyanohydrin synthesis. The highly (*S*)-selective hydroxynitrile lyase from *Manihot esculenta* (*Me*HNL) has been chosen for the conversion of acetophenone and the corresponding derivatives, which are substrates that exhibit only low grades of conversion also with several other hydroxynitrile lyases. With organic solvent-free systems under optimized reaction conditions conversions up to 78% with >99.0 *ee* (*S*) were obtained. Finally, 5 mL of (*S*)-acetophenone cyanohydrin with an enantiomeric excess of 98.5% *ee* (*S*) were synthesized.

Keywords: acetophenone cyanohydrin; enzyme catalysis; hydroxynitrile lyase; *Manihot esculenta*; thermodynamics

Introduction

Chiral cyanohydrins are versatile intermediates in the synthesis of α-hydroxy acids, β-amino alcohols, amino nitriles, α-hydroxy ketones and aziridines.[1–4] Among the known methods for the synthesis of enantiomerically pure cyanohydrins, the use of hydroxynitrile lyases for the synthesis of cyanohydrins is currently the most effective approach for this class of compounds.[5,6] These hydroxynitrile lyases have been intensively studied during the last decades with excellent results for a variety of unnatural substrates yielding (*R*)- and (*S*)-cyanohydrins in high purities and high enantiomeric excess.[7–10] An interesting member of this group is the hydroxynitrile lyase from *Manihot esculenta* (E.C. 4.1.2.37) (*Me*HNL), which exhibits high enantioselectivity for a broad spectrum of substrates of aldehydes and ketones.[11–13] Moreover, the availability of several mutants is also beneficial due to the conversion of substrates with very bulky substituents, for example, 3-phenoxybenzaldehyde (mutant *Me*HNL-W128A).[14]

Unfortunately, low grades of conversion are still observed for many substrates using both non-enzymatic and enzymatic techniques. In the field of enzymatic cyanohydrin synthesis several ketone substrates showed, for example, low conversions using several sources of hydroxynitrile lyases.[12,15,16] On the one hand, this behavior is often explained by low reactivities of ketone substrates. On the other hand, other ketone cyanohydrins are obtained with high yields and excellent enantiomeric excess, for example, phenylacetone cyanohydrin with a yield of 97% and an enantiomeric excess of 98% *ee* (*S*) using an immobilized hydroxynitrile lyase from *Manihot esculenta* (*Me*HNL).[17] This indicates that the limitations in enzymatic ketone cyanohydrin synthesis are much more complicated.

By contrast, several non-enzymatic pathways were developed to overcome these limitations, for example, catalytic asymmetric cyanosilylation using titanium isopropoxide and trimethylsilyl cyanide as a cyanide donor.[18–20] However, the requirement of anhydrous conditions, including high pressure reactions conditions, and an additional hydrolysis step represents a significant drawback, which is directly related to high costs and low atomic efficiencies. Therefore, the enzy-

matic cyanohydrin synthesis is still the best pathway to obtain enantiopure cyanohydrins.

To describe limitations in enzymatic ketone cyanohydrin synthesis we have chosen acetophenone and the corresponding derivatives as substrates, which showed low grades of conversion and only moderate enantioselectivities with several hydroxynitrile lyases.[12,16,21–23] Enantiopure acetophenone cyanohydrin is a very useful building block in the atrolactic acid synthesis.[24,25]

Results and Discussion

In biocatalytic reactions several limitations, like substrate or product inhibition, may occur, which directly lead to a limitation in productivity. On the one hand, one way to overcome the problem of inhibition is the right choice of reactor type, where a substrate inhibition can be overcome, for example, by a fed-batch process or by performing the reaction in a continuously operated stirred-tank reactor (CSTR). In case of a product inhibition an *in situ* product removal would be the best choice. Usually aqueous/organic two-phase systems and XAD-resins (for adsorption of the product) are used for this approach.[26] Additionally, thermodynamic limitations can by overcome be the removal of the product.

On the other hand, the hazardous properties of hydrogen cyanide allow for large-scale applications usually only simple batch reactions. Only small-scale applications, for example, small continuously operated membrane reactors, have proven their practicability under the safety circumstances of hydrogen cyanide.[27] Additionally, a continuous removal of the product (cyanohydrin) is not known so far in enzymatic cyanohydrin synthesis.

Finally, the undesired chemically side reaction (non-enzymatic formation of racemic cyanohydrins) can be reduced by decreasing the pH value within the aqueous phase[27,44] or by decreasing the reaction temperature.[28] The mass transfer limitation is also a powerful tool to enhance the enantiomeric excess.[29,30]

In order to understand the underlying limitations within hydroxynitrile lyase-catalyzed ketone cyanohydrin synthesis, we investigated several different reaction systems to overcome the limitations (Figure 1).

Figure 1. (*S*)-Acetophenone cyanohydrin synthesis using the hydroxynitrile lyase from *Manihot esculenta* (*Me*HNL).

Homogeneous Aqueous-Ethanol System

At the beginning, a one-phase-system with ethanol as co-solvent (to increase the solubility of acetophenone) was chosen for the determination of possible substrate and product inhibitions. However, first results showed clearly that an inhibition of the enzyme is not the limiting effect. On the contrary, the unfavorable position of the thermodynamic equilibrium prohibits high grades of conversions, which can be explained by a higher stability of the substrate acetophenone compared to the product acetophenone cyanohydrin. Within the homogenous aqueous-ethanol system, the initial substrate concentration is directly correlated with the thermodynamic equilibrium. An acetophenone concentration of only 10 mmol/L yields only a very low grade of conversion, while an initial acetophenone concentration of 500 mmol/L allows the highest grade of conversion (Figure 2). This increase of the thermodynamic equilibrium by an increase of the substrate concentration can be explained by the principle of Le Châtelier. In this case, 2 molecules (the substrates: acetophenone and hydrogen cyanide) are transformed into 1 molecule (the product:

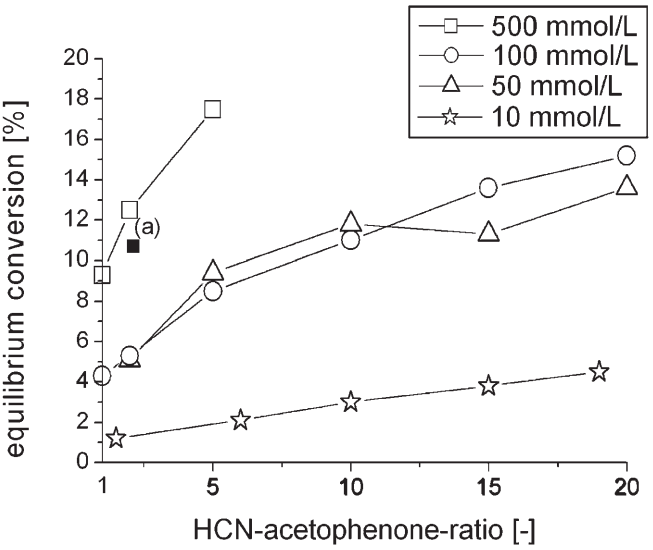

Figure 2. Acetophenone cyanohydrin synthesis in one-phase-system consisting of a mixture of ethanol and citrate buffer pH 4.0: (-☆-) 10 mmol/L acetophenone: 100% (v/v) buffer, (-△-) 50 mmol/L acetophenone: 25% (v/v) ethanol, 75% (v/v) buffer, (-○-) 100 mmol/L acetophenone: 35% (v/v) ethanol, 65% (v/v) buffer, (-□-) 500 mmol/L acetophenone 50% (v/v) ethanol, 50% (v/v) buffer. *Reaction conditions:* 5 °C, 50–500 mmol/L citrate buffer pH 4.0/ethanol, 400 rpm. Equilibrium constant: K = 0.0006 L/mmol. Due to the determination of the thermodynamic equilibrium no enzyme was required (reaction yielding the racemate). [a] [Bühler et al. 2003][12] – 200 mmol/L acetophenone, 400 mmol/L hydrogen cyanide (ratio 1:2) in diisopropyl ether with immobilized *Me*HNL on nitrocellulose (pure organic *one-phase* system).

Table 1. Enzyme stability and enzyme activity with the addition of ethanol at 5 °C.[a]

Addition of ethanol [(v/v) %]	Enzyme stability, half life period [h]	Relative enzyme activity [%]
0	>500	100.0
5	>500	18.1 ± 3.8
10	>500	17.3 ± 1.2
15	n.d.	16.1 ± 2.5
25	40	8.0 ± 3.5
50	<1	9.6 ± 1.9

[a] *Reaction conditions:* 5 °C, 50-500 mmol/L citrate buffer pH 4.0, 400 rpm; n.d. - not determined

acetophenone cyanohydrin), which is a decrease of the overall concentration of the reactants. Therefore, the reaction system responds with an increase of the equilibrium conversion when the acetophenone concentration is increased. This behavior was also found with the (better soluble) substrate benzaldehyde.[31]

Interestingly, Bühler et al. reported similar results by using pure organic one-phase systems, whereas the enzyme was immobilized on nitrocellulose (Figure 2).[12] By using an HCN to acetophenone ratio of only 2 and an initial acetophenone concentration of 200 mmol/L, 13 % conversion was obtained, which correlates with our findings. Some authors discussed a difference of the equilibrium constants between enantioselective and racemic cyanohydrin formation.[28]

On the other hand, the high concentration of ethanol has a negative effect on the enzyme stability and activity (Table 1). With increasing amount of ethanol the enzyme stability decreases dramatically from a half life period of over 500 h to only 40 h at 25 % (v/v) and less than 1 hour at 50 % (v/v) ethanol. Unfortunately, due to the extremely low enzyme stability at an ethanol content of 50 % (v/v), the enantioselective catalysis is completely suppressed and only the non-enzymatic addition of hydrogen cyanide occurs, yielding the racemate.

Two-Phase System

Two-phase systems are well established and investigated within hydroxynitrile lyase-catalyzed reactions using a variety of substrates and hydroxynitrile lyases from several sources.[29,32–34] Unfortunately, only low grades of conversion were obtained for acetophenone cyanohydrin synthesis by using a two-phase system consisting of diisopropyl ether as the organic phase and citrate buffer as the aqueous phase. Even an increase of the substrate concentration leads only to a slight increase of the equilibrium conversion (Table 2).

Table 2. Acetophenone cyanohydrin synthesis in a two-phase system.[a]

Overall acetophenone concentration within the two-phase system (DIPE buffer)	Equilibrium conversion [%]
10 mmol/L acetophenone	2.0
100 mmol/L acetophenone	2.5

[a] *Reaction conditions:* substrate to HCN ratio 1:2; diisopropyl ether to buffer ratio 1:1; citrate buffer pH 4.0; 10 °C, 400 rpm, 20 U mL^{-1} *Me*HNL

This very low equilibrium conversion is associated with the results from the homogeneous aqueous-ethanol system. Due to the high partition coefficient of acetophenone, the acetophenone concentration within the aqueous phase is very low (both experiments <5 mmol/L). Hence, these low substrate concentration result in low grades of conversion for the two-phase system.

Organic Solvent-Free System

In contrast to the one- and two-phase systems an organic solvent-free system consists only of the substrate, which represents in this special case also the organic phase, and the aqueous phase, containing the hydroxynitrile lyase and the buffer salts.

By using this unusual reaction system the equilibrium conversion can be enhanced significantly. At a fixed acetophenone to buffer ratio of 1:20 (equivalent to 0.41 mol/L acetophenone) and an increasing acetophenone to HCN ratio up to 1:20, the thermodynamic equilibrium conversion has been raised up to 46 % (Table 3). Unfortunately, the enzyme stability decreases simultaneously with increasing hydrogen cyanide and acetophenone concentrations from >500 h in pure buffer (Table 1) *via* 29 h in the organic solvent free system with acetophenone and finally to 7 h with 2 mol/L hydrogen cyanide (also organic solvent-free system with an acetophenone to HCN ratio of 1:5). This prevents high grades of enantioselective conversion. The same behavior was also found for the closely related hydroxynitrile lyase from *Hevea brasiliensis*.[35–37]

Nevertheless, for an acetophenone to HCN ratio of 1:5 an enzyme stability of 7 h still enables 22 % conversion with excellent 97 % *ee* (*S*). Higher hydrogen cyanide concentrations permanently deactivate the enzyme and thus prevent higher grades of conversion, which is a general problem in hydroxynitrile lyase-catalyzed cyanohydrin synthesis.[37,38]

Furthermore, the acetophenone to buffer ratio has also a significant influence on the equilibrium conversion. By adjusting the acetophenone to buffer ratio

Table 3. Variation of acetophenone to HCN ratio.[a]

Acetophenone to HCN ratio	Enzyme stability (half life time) [h]	Equilibrium conversion [%]	pH 4.8		pH 4.0	
			Conversion [%]	Enantiomeric excess [%]	Conversion [%]	Enantiomeric excess [%]
1:0	29	0	0	0	0	0
1:2	8	10	10	95	n.d.	-
1:5	7	22	22	96	22	97
1:10	<0.5	31	6	6	22	97
1:15	<0.5	37	5	0	0	0
1:20	n.d.	46	5	0	0	0

[a] *Reaction conditions:* reaction time: 6 h; acetophenone to buffer ratio 1:20 (overall acetophenone concentration equivalent to 0.41 mol/L), citrate buffer pH 4.0 or pH 4.8, 5 °C, 400 rpm; 450 U mL^{-1} for pH 4.0; 150 U mL^{-1} for pH 4.5; n.d. = not determined.

Table 4. Variation of acetophenone to buffer ratio.[a]

Acetophenone to buffer ratio	Overall acetophenone concentration [mol/L]	pH 4.8; 150 U mL^{-1} *Me*HNL		pH 4.0; 450 U mL^{-1} *Me*HNL	
		Conversion [%]	Enantiomeric excess [%]	Conversion [%]	Enantiomeric excess [%]
1:100	0.085	n.d.	-	9	86
1:33	0.25	11	95	11	94
1:20	0.41	22	96	22	97
1:10	0.78	36	91	15	98
1:6.6	1.13	31	75	n.d.	n. d.

[a] *Reaction conditions:* reaction time: 6 h; acetophenone to HCN ratio 1:5; citrate buffer pH 4.0 or pH 4.8, 5 °C, 400 rpm; n.d. = not determined.

from 1:100 up to 1:10 the equilibrium conversion has been increased from 9% up to 36% with a slight decline of the enantioselectivity (Table 4).

Noteworthy, the hydrogen cyanide concentration increases simultaneously with the increasing acetophenone concentration at a constant acetophenone to HCN ratio of 1:5. This leads again to a loss of enzyme stability and prevents higher grades of conversion at even lower acetophenone to buffer ratios (1:6.6).

Reactions with Acetophenone Derivatives

Finally, the position and kind of substituents at the aromatic ring also influence the maximal conversion and enantiomeric excess within hydroxynitrile lyase-catalyzed reactions.[39] Therefore, several acetophenone derivatives were tested under the optimized conditions, but only substrates being that were liquid at 5 °C were applied.

On the one hand, substrates with electronegative substituents like fluoro, chloro and nitro groups showed the best results, for example, yields up to 71% could be obtained for the substrate 2′-fluoroacetophenone (Table 5). Additionally, the position of the substituents also displays a significant influence on the maximum conversion. For instance, 4′-fluoroacetophenone was converted to the corresponding cyanohydrin only in 14% yield, while 2′-fluoroacetophenone showed a conversion of 71% (as already stated above). This can be explained by an intramolecular hydrogen bond, which stabilizes the cyanohydrin and facilitates high grades of conversion (Figure 3).[40] In contrast, by applying a two-phase system (diisopropyl ether-citrate buffer) for 2′-fluoroacetophenone the conversion decreases to 8%, the effect was pointed out in the section 'Two-Phase System'. The intramolecular stabilization by a hydrogen bond occurs also for 2-nitroacetophenone cyanohydrin. In contrast to this, 2′-methoxyacetophenone cyanohydrin and 2′-hydroxyacetophenone result still only in low grades of conversion. Potentially, the positive mesomeric effect of the oxygen atom is still too strong and thus prevents higher equilibrium conversions.

Additionally, the conversions of 2′-chloroacetophenone and 2′-bromoacetophenone result in lower yields and enantiomeric purities, which can be explained by steric effects due to the size of the substituent.

On the other hand, electropositive substituents like methyl and amino groups resulted in extremely low conversions of less than 1%. 2′,3′,4′,5′,6′-Fluoroacetophenone affords also a very low conversion, while the reasons for this behavior are not completely known till now.

Table 5. Acetophenone derivative cyanohydrin formation.[a]

Acetophenone (AP) derivative	Time [h]	Conversion [%]	*ee* (*S*) [%]
AP	6	22	96
4′-F-AP	1.5	14	>99
3′-F-AP	**3**	**48**	**>99**
2′-F-AP	**3**	**71**	**>99**
2′,3′,4′,5′,6′-F-AP	6	<1	-
4′-Cl-AP	6	18	97
3′-Cl-AP	**6**	**23**	**97**
2′-Cl-AP	4.5	6	80
4′-Br-AP	solid		
3′-Br-AP	6	10	>99
2′-Br-AP	6	9	68
4′-I-AP	solid		
2′-I-AP	6	<1	-
4′-Me-AP	6	<1	-
3′-Me-AP	6	<1	-
2′-Me-AP	6	<1	-
4′-MeO-AP	solid		
3′-MeO-AP	6	<1	-
2′-MeO-AP	6	<1	-
4′-NO_2-AP	solid		
3′-NO_2-AP	solid		
2′-NO_2-AP	**1.5**	**40**	**>99**
4′-NH_2-AP	solid		
3′-NH_2-AP	solid		
2′-NH_2-AP	6	<1	-
4′-OH-AP	solid		
3′-OH-AP	solid		
2′-OH-AP	6	<1	-

[a] *Reaction conditions:* reaction time: 1.5–6 h; 0.4 mmol acetophenone derivative: 2 mmol hydrogen cyanide; 1 mL citrate buffer pH 4.0, 5 °C, 450 U mL^{-1}, 400 rpm; solid = not determined due to the absence of an organic layer.

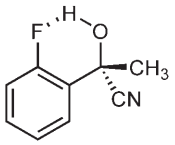

Figure 3. Possible intramolecular hydrogen bond of (*S*)-2′-fluoroacetophenone.

Conclusions

We investigated the use of organic solvent-free systems for their practicability to overcome limitations and bottlenecks within acetophenone cyanohydrin synthesis. We pointed out that organic solvent-free systems enable good conversions also for substrates which otherwise showed unsatisfactory results using conventional methods, like aqueous-organic two-phase systems.

The organic solvent free system has several advantages.

Firstly, the substrate concentration within the aqueous phase correlates directly with the enzyme activity. Especially at substrate concentrations below the K_M value (Michaelis–Menten constant) the enzyme activity will be reduced dramatically. In the case of an aqueous organic two phase-system, for example, with diisopropyl ether as the organic solvent and a buffer as the aqueous layer, the partition coefficient of the substrate generates only a low substrate concentration within the aqueous phase, which leads directly to a low enzyme activity. The kinetics within the two-phase system were intensely studied during the last years.[33,36] On the other hand, the organic solvent-free system affords always a maximum enzyme activity, because the substrate concentration is only limited by the solubility of the substrate in the aqueous phase.

Secondly, an increase of the substrate concentration within the aqueous phase results also in higher grades of conversion, as pointed out in the section 'Homogenous Aqueous-Ethanol System'. Therefore the organic solvent-free system is extremely advantageous, because the substrate concentration is only limited by the solubility of the substrate, resulting in very high grades of conversion.

Thirdly, the organic layer (in this case mainly the substrate) acts for the product (cyanohydrin) as an ordinary organic phase, resulting in a simple partition coefficient of the product. Therefore the product is selectively extracted into the organic phase, whereas the substrate concentration in the aqueous phase (where the enzymatic reaction takes place) is still maximal due to the limited water miscibility of acetophenone. This *in situ* product removal shifts the equilibrium to higher grades of conversion, depending also on the type and position of the substituents.

Which effect is more relevant to explain the observed higher conversion can only be confirmed with a thorough investigation. This would include exact determination of phase ratios, distribution coefficients and equilibrium constants, but this is complicated due to the volatility of hydrogen cyanide. That such studies are indeed possible has been shown recently for the enantioselective ketone reduction using alcohol dehydrogenases.[41,42]

The potential of the approach presented here was proven by synthesis of 5 mL of (*S*)-acetophenone cyanohydrin with an enantiomeric excess of 98.5% *ee* (*S*). This included also a distillation step without any decomposition or racemization of the product.[43]

In summary, the organic solvent-free system enables moderate to high conversions, even for substrates which were not obtained by conventional reaction systems, for example, the two-phase system using diisopropyl ether and buffer or immobilized enzyme in diisopropyl ether. Unfortunately, the enzyme stability is reduced by the very high concentrations of acetophenone and hydrogen cyanide, but the hydroxynitrile

lyase from *Manihot esculenta* still exhibited good enzyme stability and excellent enantioselectivity.

Experimental Section

Chemicals

2′-Methylacetophenone, 2′-fluoroacetophenone, 2′-iodoacetophenone, 2′-nitroacetophenone, 3′-chloroacetophenone and 3′-methylacetophenone were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. 2′-Hydroxyacetophenone, 2′-bromoacetophenone, 3′-methoxyacetophenone, 3′-fluoroacetophenone, 3′-bromoacetophenone, 4′-fluoroacetophenone and 2′,3′,4′,5′,6′-pentafluoroacetophenone were obtained from Acros Organics, Geel, Belgium. 2′-Chloroacetophenone, 4′-chloroacetophenone, racemic mandelonitrile and acetophenone were products of Merck-Schuchardt, Hohenbrunn, Germany. 4′-Methylacetophenone, 2′-methoxyacetophenone and sodium cyanide were purchased from Fluka Chemie GmbH, Buchs, Switzerland.

Enzyme

The (*S*)-hydroxynitrile lyase from *Manihot esculenta* (recombinant in *E. coli*) was obtained from Julich Chiral Solutions GmbH (http://www.julich.com/), Jülich, Germany.

Enzyme Assay

The enzyme activity was determined by following the cleavage of *rac*-mandelonitrile into benzaldehyde and HCN at 25 °C. The formation of benzaldehyde was measured spectrometrically at 280 nm. The non-enzymatic cleavage reaction was monitored under identical conditions and subtracted. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the cleavage of 1 μmol mandelonitrile per minute under assay conditions.

Assay conditions: 700 μL citrate-phosphate buffer pH 5.0, 100 μL enzyme solution (dilution if required) and 200 μL mandelonitrile stock solution (60 mmol/L in citrate-phosphate pH 3.5) were mixed in a cuvette with 1 cm pathlength and the increase of absorbance at 280 nm was measured for 2 min.

HCN Formation, General Procedure

The required amount of HCN was freshly distilled in a well ventilated hood. 4 grams of sodium cyanide were dissolved in 10 mL de-ionized water and 10 mL of 5 mol/L sulfuric acid were added dropwise within 2 min. Afterwards the combined solutions were heated up to 75 °C and formed HCN was trapped and stored at 5 °C. For the removal of traces of water traces a spatula tip of sodium sulfate was added. All waste solutions were collected and disposed.

An electrochemical HCN detector (Micro III G203, GfG-Gesellschaft für Gerätebau mbH, Dortmund, Germany) was placed in the hood for continuous monitoring.

Reaction Conditions for the Enzymatic Synthesis of Cyanohydrins

All reactions were carried out at 5 °C.

Homogenous Aqueous-Ethanol System

The required amount of ethanol (co-solvent) was added to 50–500 mM citrate buffer pH 4.0 in order to obtain the desired solubility of acetophenone in buffer. Afterwards acetophenone and hydrogen cyanide were added. Due to the determination of the thermodynamic equilibrium *no enzyme* was required.

Two-Phase System

The required amounts of acetophenone and hydrogen cyanide were added to the biphasic system, consisting of diisopropyl ether and 50–500 mM citrate buffer (pH 4.0, ratio 1:1). The enzymatic reaction was started by the addition of the enzyme solution (20 U mL^{-1}).

Organic Solvent-Free System, General Procedure

Acetophenone and hydrogen cyanide were added to 1 mL buffer, the reaction was initiated by the addition of the enzyme solution (450 U mL^{-1}).

Comparison of the Acetophenone Derivatives

0.4 mmol of the acetophenone derivative and 2 mmol of hydrogen cyanide were added to 1 mL 50–500 mM citrate buffer (pH 0.0 or pH 4.8). The enzymatic reaction was started by the addition of the required enzyme solution (150 or 450 U mL^{-1}).

Sample Taking

Homogeneous aqueous-ethanol system: A sample (100 μL) was extracted with 100 μL *n*-hexane. 50 μL of the organic phase (*n*-hexane) were added to a mixture of 500 μL dichloromethane, 50 μL trifluoroacetic anhydride and 50 μL pyridine for the acetylation procedure.

Two-phase system: 50 μL of the organic phase were added to a mixture of 500 μL dichloromethane, 50 μL trifluoroacetic anhydride and 50 μL pyridine for the acetylation procedure.

Organic solvent-free system: A sample (100 μL) of the suspension was added to 100 μL of diisopropyl ether for the extraction. 50 μL of the organic phase were added to a mixture of 500 μL dichloromethane, 50 μL trifluoroacetic anhydride and 50 μL pyridine for the acetylation procedure.

GC Analysis for Determination of Conversion and Enantiomeric Excess

The conversion of acetophenone (and the derivatives) to acetophenone cyanohydrin (derivatives) as well as their enantiomeric excess were determined by gas chromatographic analysis with a Chiraldex capillary gas chromatography column (G-PN – γ-Cyclodextrin, Propionyl) from astec using a CP3800 (Varian) with a flame ionization detector (FID). Carrier gas was helium at 2 mL min^{-1}. Temperature gradient: 80 °C for 0.5 min, rise with 10 °C min^{-1} to 130 °C and hold 130 °C for 15 min . The injector and detector temperatures were set to 250 °C.

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Publikation 3

“A new (R)-selective Hydroxynitrile Lyase from Arabidopsis thaliana with an alpha/beta-Hydrolase fold”

Andexer, J.; **von Langermann, J.**; Mell, A.; Bocola, M.; Kragl, U.; Eggert, T.; Pohl, M.

Angewandte Chemie - International Edition, 46, 8679-8681 (2007)

Anteile: Andexer, J. (30%); **von Langermann, J. (20%)**; Mell, A. (15%); Bocola, M. (20%); Kragl, U. (5%); Eggert, T. (5%); Pohl, M. (5%)

Einleitung zu Publikation 3

Die industrielle Synthese von (*S*)-Cyanhydrinen ist mittlerweile hervorragend durch die (*S*)-selektiven Hydroxynitril Lyasen aus *Manihot esculenta* und *Hevea brasiliensis* bzw. durch entsprechende Mutanten abgedeckt. Dagegen ist für die Synthese von (*R*)-Cyanhydrinen nur durch eine enge Auswahl an (*R*)-selektiven Hydroxynitril Lyases vorhanden (z.Bsp. aus *Prunus amygdalus*, *Pa*HNL).

Eine mögliche Alternative könnte die (*R*)-Hydroxynitril Lyase aus *Arabidopsis thaliana* (*At*HNL) darstellen, da sie sowohl aliphatische als auch aromatische Substrate umsetzt. Die hieraus folgende Aufgabenstellung war somit die Wahl eines geeigneten Reaktionsmediums zur Durchführung des Substratscreenings und die Optimierung der Reaktionsparameter (Abbildung 8-3).

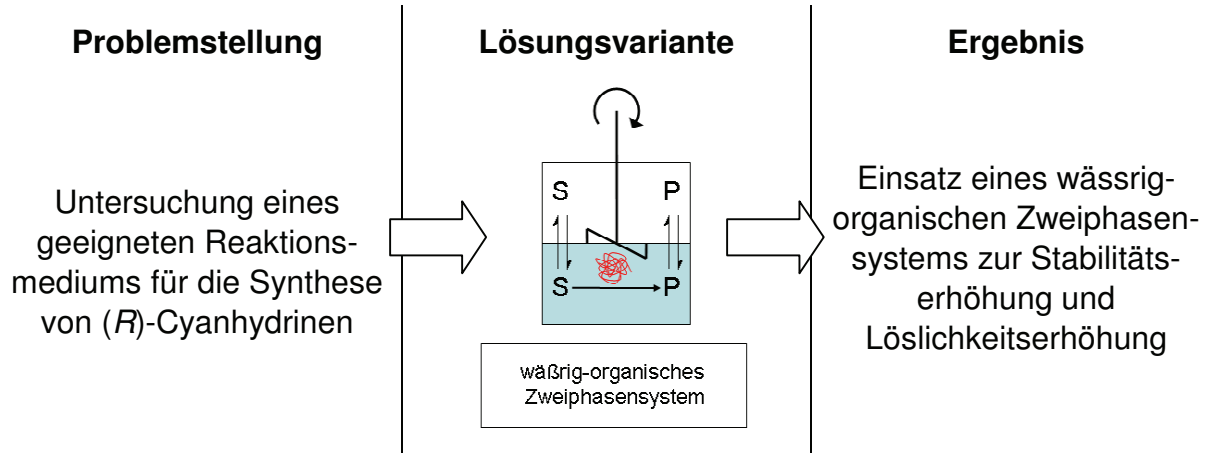

Abbildung 8-3: Vorgehensweise Publikation 3

Erstaunlicherweise zeigt diese Hydroxynitril Lyase eine außerordentlich hohe (*R*)-Selektivität, welche fast das gesamte Substratspektrum umfasst. Durch die Verwendung des klassischen wässrig-organischen Zweiphasensystems (Diisopropylether-Puffer) konnten darüber hinaus akzeptable Aktivitäten erzeugt werden. Reduziert wird die Leistungsfähigkeit der *At*HNL momentan (noch) durch die geringe Stabilität des Enzyms unter Reaktionsbedingungen.

Enzyme Catalysis

DOI: 10.1002/anie.200701455

An *R*-Selective Hydroxynitrile Lyase from *Arabidopsis thaliana* with an α/β-Hydrolase Fold**

Jennifer Andexer, Jan von Langermann, Annett Mell, Marco Bocola, Udo Kragl, Thorsten Eggert,* and Martina Pohl**

Hydroxynitrile lyases (HNLs) catalyze the stereoselective formation of C–C bonds between HCN and aldehydes or ketones yielding chiral cyanohydrins, which are versatile building blocks for the pharmaceutical and agrochemical industries.[1] Among the most important cyanohdrins are chiral α-hydroxy acids such as substituted mandelic acids,[1e,f,2a] *m*-phenoxybenzaldehyde derivatives,[2c] and structures with additional aliphatic linkers between the aldehyde moiety and aromatic ring which are useful for the synthesis of "prils".[2d] In nature HNLs catalyze the cleavage of cyanohydrins, known as cyanogenesis. The currently known HNLs can be divided into two groups: *R*-selective enzymes evolved from oxidoreductase ancestors, such as HNLs from various *Rosaceae*[2] and from *Linum usitatissimum*,[3a] and *S*-selective enzymes derived from hydrolases with an α/β-hydrolase fold; these encompassing the enzymes from *Hevea brasiliensis* (*Hb*HNL),[3b] *Manihot esculenta* (*Me*HNL),[3c] and *Sorghum bicolor* (*Sb*HNL).[3d] Here we present the first exception to this accepted rule with the first *R*-selective HNL containing an α/β-hydrolase fold from the noncyanogenic plant *Arabidopsis thaliana* (mouse-ear cress).

Owing to the growing demand for chiral compounds like cyanohydrins there is a strong motivation to identify new stereoselective HNLs with a broad substrate range which can be easily and economically produced. These demands are fulfilled by the currently available *S*-selective enzymes *Hb*HNL and *Me*HNL: they can be expressed in bacterial hosts like *Escherichia coli* and accept a broad range of aromatic and aliphatic aldehydes as well as ketones.[4] A similar broad substrate range has been reported for the *R*-selective HNLs isolated from some *Prunus* species (*P. amygdalus* (*Pa*HNL) and *P. mume* (*Pm*HNL)). These biocatalysts are either used as defatted seed meals or, in the case of *Pa*HNL (isoenzyme 5), are expressed in the yeast *Pichia pastoris*.[2a,e]

Recently, several approaches were reported to identify new HNLs for biocatalytic processes by screening different cyanogenic plant extracts for HNL activity, yielding some new enzyme sources.[5] Attempts to identify new enzymes based on sequence similarities to known HNLs have not yet been successful.[6,7]

Several sequences similar to *Me*HNL and *Hb*HNL are found in the genome of the noncyanogenic model plant *Arabidopsis thaliana*.[7] In the course of our studies on structure–function relationships of α/β-hydrolases we cloned several genes encoding *Arabidopsis* proteins with high sequence similarity to *Me*HNL and *Hb*HNL and expressed them in *E. coli*. Unexpectedly, one of them (gene bank entry: AAN13041) shows high activity towards mandelonitrile and catalyzes also the cleavage of some other cyanohydrins derived from cyclohexanone and *m*-phenoxybenzaldehyde, while acetaldehyde, propionaldehyde, and acetone cyanohydrin are poor substrates.[8]

A subsequent investigation of the cyanohydrin-forming activity revealed that the new enzyme is highly *R*-selective with a broad substrate range including various aromatic and aliphatic aldehydes as well as ketones, which are converted to *R*-cyanohydrins with good to excellent yields and mainly excellent enantioselectivities (Table 1).[9] As can be seen, a whole range of substituted benzaldehydes are converted with excellent activity and enantioselectivity. There was no optimization of the reaction time, but substrates such as **3**, **4**, and **6**, which react even in the absence of the enzyme, gave products with 99% *ee* indicating a high enzymatic activity towards these substrates. To obtain complete conversion of substrates with the more bulky substituents the reaction time had to be increased slightly. It should be also noted that the reaction was performed at pH 5. Lowering the pH could of course suppress the nonenzymatic reaction even further. But even at pH 5 the *ee* obtained is higher for *o*-chlorobenzaldehyde cyanohydrin than that in earlier studies with optimized *Pa*HNL[2a] or with the wild-type enzyme.[10] Subsequent hydrolysis yields (*R*)-*o*-chloromandelic acid, which is a key

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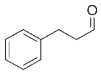
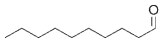
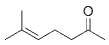
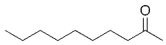
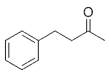
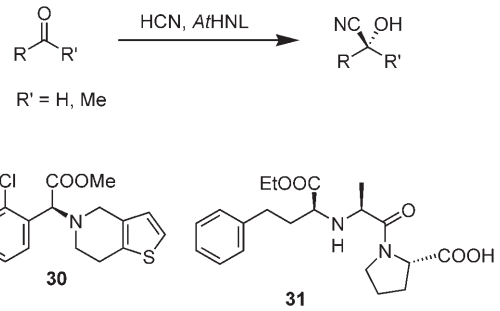
[**] The authors thank the group of Ute Höcker (University of Düsseldorf, Botanik IV) for providing *Arabidopsis* cDNA and mRNA.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

Table 1: Substrate range of *At*HNL.[a]

Substrate		t [h]	X_{enz}[b] [%]	*ee* (*R*) [%]	X_{nenz}[b] [%]
					
R = H	**1**	2	> 99	> 99	14
R = *o*-F	**2**	2	> 99	99	17
R = *o*-Cl	**3**	2	> 99	99	26
R = *o*-Br	**4**	6	99	98	42
R = *o*-I	**5**	3	> 99	> 95	26
R = *m*-F	**6**	2	> 99	> 99	22
R = *m*-Cl	**7**	3	99	> 99	7
R = *m*-Br	**8**	6	99	95	9
R = *m*-I	**9**	6	98	93	5
R = *m*-PhO	**10**	22	83	> 95	0
R = *p*-F	**11**	2	> 99	> 99	7
R = *p*-Cl	**12**	2	> 99	> 99	4
R = *p*-Br	**13**	3	99	> 99	4
R = *p*-I-	**14**	6	99	92	7
R = *p*-OH-	**15**	3	96	97	3
R = *p*-OMe-	**16**	22	87	68	14
	**17**	22	97	96	97
	**18**	22	99	68	97
	**19**	6	68	n.d.[c]	6
	**20**	6	99	98	78
	**21**	22	56	> 95	0
	**22**	3	53	n.d.[c]	0
	**23**	22	0	–	0
	**24**	6	48	95	2
	**25**	22	2	–	0
	**26**	3	94	–[d]	76
	**27**	22	7	n.d.	0
	**28**	22	8	95	0
	**29**	3	1	–	0

[a] All conversions were performed in a two-phase system; conversion (*X*) and enantiomeric excess (*ee*) were determined by gas chromatography.[9] n.d. = not determined. [b] enz: enzymatic; nenz: nonenzymatic. [c] Separation of enantiomers by the Chiraldex capillary GC column (G-PN-γ-cyclodextrin, propionyl) was not possible. [d] Achiral product.



Scheme 1. *At*HNL-catalyzed synthesis of chiral cyanohydrins and examples of compounds obtained after subsequent reactions.

intermediate for the antithrombotic agent clopidogrel (**30**). The cyanohydrin of **18** can be transferred to the corresponding α-hydroxyester, which is a building block of ACE inhibitors such as enalapril (**31**; Scheme 1).

The reaction of substrate **18** is somewhat less selective than that of **17**, indicating that the enzymatic reaction is slower and therefore the reaction conditions, primarily the pH, must be fine-tuned. Also an increasing chain length of the aliphatic aldehydes reduces the activity but not the stereoselectivity. In comparison, the enzyme is less active towards aliphatic and aromatic ketones.

In order to rationalize similarities and differences concerning the reaction mechanism and stereoselectivity of *At*HNL relative to the structurally similar, but *S*-selective *Hb*HNL and *Me*HNL, a homology model was created, based on the crystal structures of *Hb*HNL.[9,11] A comparison of both structures suggests a typical catalytic triad consisting of Ser 81, Asp 208, and His 236 also in *At*HNL (Figure 1).

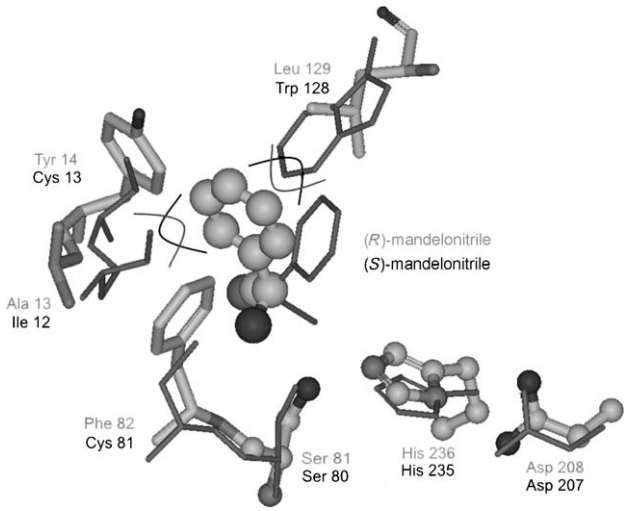


Figure 1. Overlay of the crystal structure of *Hb*HNL (dark gray, thin lines)[11] and the structural model of *At*HNL (light gray, thick rods). The catalytic triad (Ser/His/Asp) and the residues in contact with bound mandelonitrile are shown: (*R*)-mandelonitrile in the *At*HNL model and (*S*)-mandelonitrile in the crystal structure of *Hb*HNL (1YB8). *At*HNL reveals a specific binding pocket for (*R*)-mandelonitrile between Leu 129 and Ala 13 which is blocked by Trp 128 and Ile 12 in *Hb*HNL.

These residues were exchanged by nonfunctional, but sterically similar residues using site-directed mutagenesis, and the resulting variants (Ser81Ala, Asp208Asn, His236Phe) showed drastically impaired catalytic activity (< 2 %), supporting their catalytically important function.[9] A further catalytically important residue (Lys 236), which has been identified in *Hb*HNL,[11a] is replaced by Met 237 in *At*HNL.

To analyze the differences in stereoselectivity a structural model of *At*HNL with (*R*)-mandelonitrile bound to the active

site was created based on the structure of *Hb*HNL containing (*S*)-mandelonitrile.[9,11] In comparison to *Hb*HNL, two side chains of the potential substrate-binding pocket in *At*HNL are exchanged. These are Trp128 and Cys13 in *Hb*HNL, which are replaced by Leu129 and Tyr14, respectively, in *At*HNL (Figure 1). The strict *S* selectivity of *Hb*HNL can be understood from the constructed model, since Trp128 and Ile12 sterically hinder the binding of (*R*)-mandelonitrile. On the other hand, it can be expected that the aromatic side chains of Tyr14 and Phe82 might stabilize (*R*)-mandelonitrile in the binding pocket of *At*HNL.

In first experiments with an *At*HNL variant (Tyr14Cys) still exclusively (*R*)-mandelonitrile was produced, suggesting that a single exchange is not sufficient to alter the stereoselectivity of *At*HNL. Studies on a double mutant (Tyr14Cys/Leu129Trp) and the crystal structure of the enzyme are in progress. The homology model is not yet accurate enough to explain the differences in activity or substrate selectivity as discussed before.

We have described a novel *R*-specific HNL (E.C. 4.2.1.–) from *Arabidopsis thaliana* and its application in biocatalytic processes. The enzyme is a good alternative to currently known *R*-selective HNLs, such as *Pa*HNL,[2e] for the production of *R*-cyanohydrins as it is readily available in technically relevant amounts by overexpression in *E. coli*. Its broad substrate range includes aliphatic and aromatic aldehydes as well as ketones.[14] As the first *R*-specific HNL based on an α/β-hydrolase fold, its structure will provide valuable information concerning the enzyme mechanism of α/β-hydrolase fold based HNLs.

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Keywords: cyanohydrins · enzyme catalysis · genetic engineering · hydroxynitrile lyases · oxynitrilases

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[8] Mandelonitrile cleavage was measured according to Bauer et al.;[12] cleavage of other cyanohydrins was detected with an HCN-based assay system.[13]

[9] For experimental details see the Supporting Information.

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[14] *At*HNL and its application have been filed for patent (J. Andexer, T. Eggert, evocatal GmbH, German patent application DE 10 2006 058 373.6, **2006**).

Publikation 4

“Hydroxynitrile lyase catalyzed cyanohydrin synthesis at high pH-values”

von Langermann, J.; Guterl, J.-K.; Pohl, M.; Wajant, H.; Kragl, U.
Bioprocess and Biosystems Engineering, 31, 155-161 (2008)

Anteile: **von Langermann, J. (70%)**; Guterl, J.-K. (15%); Pohl, M. (5%); Wajant, H. (5%); Kragl, U. (5%)

Einleitung zu Publikation 4

Die nicht-enzymatische Reaktion ist eines der wichtigsten Problemstellungen innerhalb der enzymatischen Cyanhydrinsynthese, da hierbei nur das racemische Produkt gebildet wird. Ziel der Publikation 4 war es, die Gesetzmäßigkeiten dieser unerwünschten Nebenreaktion zu charakterisieren und somit das (praktikable) Substratspektrum zu erweiteren (Abbildung 8-4).

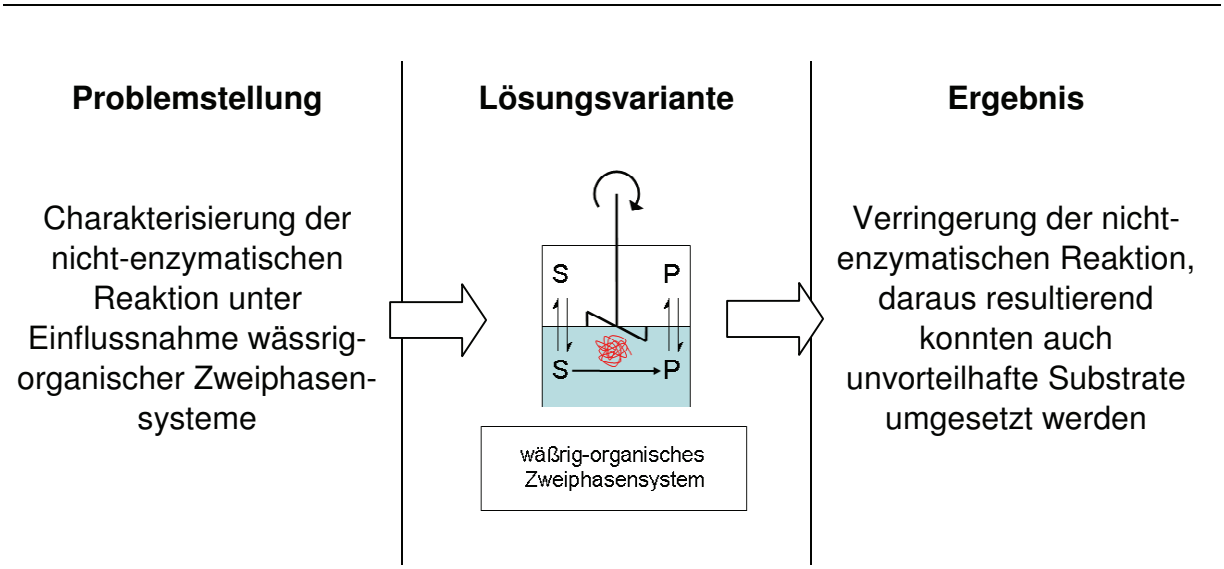

Abbildung 8-4: Vorgehensweise Publikation 4

Durch eine entsprechende Modifikation der Reaktionsbedingungen sind auch eher unvorteilhafte Substrate umgesetzt worden. Im vorliegenden Falle konnte 3-Phenoxybenzaldehyd mit der Wildtyp-Variante der Hydroxynitril Lyase umgesetzt werden, wobei im Gegensatz zu den klassischen Reaktionsbedingungen deutliche höhere Reaktivitäten und Enantioselektivitäten möglich waren.

Bioprocess Biosyst Eng (2008) 31:155–161
DOI 10.1007/s00449-008-0198-4

ORIGINAL PAPER

Hydroxynitrile lyase catalyzed cyanohydrin synthesis at high pH-values

Jan von Langermann · Jan-Karl Guterl · Martina Pohl · Harald Wajant · Udo Kragl

Received: 6 November 2007 / Accepted: 3 January 2008 / Published online: 18 January 2008

Abstract The application of unusual high pH-values within enzymatic cyanohydrin synthesis has been investigated. Usually enzymatic cyanohydrin synthesis in two-phase systems requires low pH-values within the aqueous phase to suppress the non-enzymatic side reaction. In contrast, we investigated the usage of pH-values above pH 6 by using the highly enantioselective (*S*)-selective hydroxynitrile lyase from *Manihot esculenta*. With these unusual reaction conditions also the unfavorable substrate 3-phenoxy-benzaldehyde can be converted by the wild type enzyme with excellent conversion and enantiomeric excess yielding pure (*S*)-3-phenoxy-benzaldehyde cyanohydrin with an enantiomeric excess of 97%. Although the variant *Me*HNL–W128A shows a higher activity with respect to this reaction, the enantioselectivity was reduced (85% e.e.(*S*)). Additionally, a new continuous spectroscopic cyanohydrin assay monitoring the formation of 3-phenoxy-benzaldehyde cyanohydrin was developed.

Keywords Enzyme catalysis · Cyanohydrin · Hydroxynitrile lyase · pH-value

Dedicated to Prof. Dr. Christian Wandrey on the occasion of his 65th birthday.

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Introduction

Enantiopure cyanohydrins are versatile building blocks for a broad range of further products, e.g. α-hydroxy-acids, β-amino-alcohols and aziridines [1–4]. For the synthesis of enantiopure cyanohydrins hydroxynitrile lyases (HNL) were found to be the most promising approach [5–7]. Since 1908 these enzymes have been intensively studied with excellent results also for a variety of unnatural substrates yielding enantiopure cyanohydrins in high purities and high enantiomeric excesses [8–10]. Hydroxynitrile lyases were obtained from several sources, e.g. (*R*)-selective hydroxynitrile lyases from *Prunus amygdalus*, *Linum usitatissimum* and *Arabidopsis thaliana* and (*S*)-selective hydroxynitrile lyases from *Manihot esculenta*, *Hevea brasiliensis* and *Sorghum bicolor* [11–13]. An interesting member is the (*S*)-selective hydroxynitrile lyase from *Manihot esculenta*, which exhibits a broad spectrum of aldehydes and ketones. Additionally, some variants are also available, among these the variant *Me*HNL–W128A expands the substrate range to more bulky substrate [14–16].

Several reaction systems were developed to apply hydroxynitrile lyases within organic synthesis [15]. Usually pure organic reaction systems with an immobilized hydroxynitrile lyase (e.g. on nitrocellulose) and two-phase systems with a dissolved hydroxynitrile lyase were used. Pure organic reaction systems consists of an organic

solvent, wherein the substrates and products are dissolved. Under these circumstances this reaction system acts like a one-phase-system and thus affords high grades of conversion even at low hydrogen cyanide–substrate ratios [17]. In contrast, the two-phase approach usually consist of diisopropylether as the organic layer and a buffer as the aqueous phase [18].

In fact, every reaction system including hydroxynitrile lyases consists of two parallel reactions, the enzymatic and the non-enzymatic reaction (Fig. 1). On the one hand, the enzymatic reaction is catalyzed by the enzyme yielding the enantiopure cyanohydrin.

On the other hand, the non-enzymatic reaction leads to the formation of racemic cyanohydrin, which decreases the enantiomeric excess of the final product. Therefore the ratio between the enzymatic and the non-enzymatic reaction is finally the limiting effect for the enantiomeric excess of the product (Fig. 2).

To obtain an enantiomeric enriched products relatively high ratios are required, e.g. to achieve an enantiomeric excess of 99% a ratio of 100:1 and for 99.9% a ratio of 1,000:1 is obligatory.

Therefore, the non-enzymatic must be significantly reduced, which is usually accomplished by a decrease of the pH-value in the aqueous phase and a decrease of the reaction temperature [19, 20]. The mass transfer limitation is also a powerful tool to enhance the enantiomeric excess [21, 22]. Disadvantageously, these procedures reduce also the enzyme activity and result in higher enzyme costs.

Within this report we describe the conversion of 3-phenoxy-benzaldehyde to the corresponding cyanohydrin by applying a high pH two-phase system (pH > 6). Enantiomeric pure 3-phenoxy-benzaldehydecyanhydrin is a very useful intermediate in the pyrethroid synthesis [23].

This unusual reaction system enables even the usage of the wild type *Me*HNL, which usually does only poorly accept substrates with such bulky substituents. Conversions up to 98% with an enantiomeric excess up to 97% (*S*) were possible.

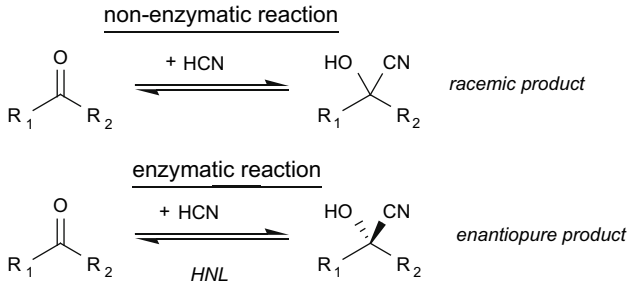

Fig. 1 Parallel cyanohydrin forming reactions in aqueous solution

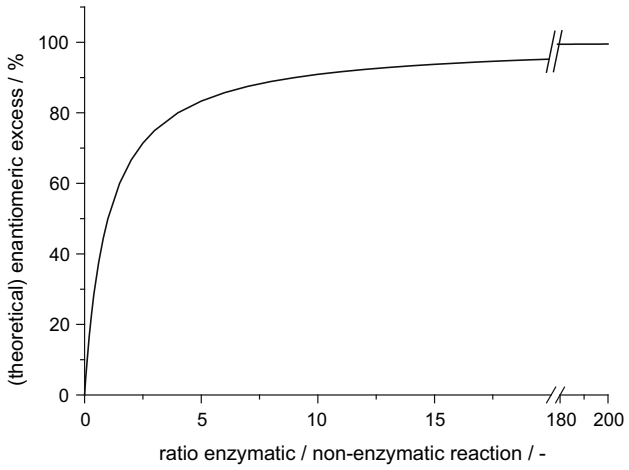

Fig. 2 Influence of the ratio enzymatic/non-enzymatic reaction on the enantiomeric excess of the product (calculated, with enzymatic reaction e.e. = 100% and non-enzymatic reaction e.e. = 0%)

Experimental

Chemicals

3-Phenoxy-benzaldehyde and sodium cyanide were purchased from Fluka Chemie GmbH, Buchs, Switzerland. The racemic mandelonitrile was received from Merck–Schuchardt, Hohenbrunn, Germany.

Enzyme

The (*S*)-hydroxynitrile lyase from *Manihot esculenta* was obtained from Julich Chiral Solutions GmbH (http://www.julich.com/), Jülich, Germany [24]. Specifications wild type: aqueous solution, partially purified; 6,382 U(mandelonitrile)/mL. Specifications variant W128A: aqueous solution, partially purified; 139 U(mandelonitrile)/mL.

Enzyme assays

Cleavage reaction (racemic mandelonitrile)

One unit of enzyme activity was defined as the amount of enzyme that catalyzes the cleavage of 1 µmol mandelonitrile per minute under assay conditions.

Eight hundred and fifty microliter of McIlvaine–citrate–phosphate buffer pH 5.4 (100 mL are prepared from 22 mL citric acid (100 mM) and 27.7 mL Na_2PO_4 (200 mM) are mixed in an Eppendorf cap with 100 µl of the enzyme sample and incubated at 25°C for 5 min in a thermoblock. Afterwards the reaction is started by the addition of 50 µl mandelonitrile solution (600 mmol/L mandelonitrile in diisopropylether) followed by intensive mixing using a

vortex mixer. Subsequently, the mixture is transferred into a quartz cuvette and the increase of the OD_{280} is measured for 2 min relative to an empty cuvette. Note that the final concentration of DIPE in the cuvette is 5% (v/v). Since DIPE is hardly soluble in aqueous systems (water solubility: 0.2% v/v), the measurements often start with a lag-phase, due to phase separation. During this time the OD_{280} does not increase or even decreases. The lag-phase may last up to 30 s and should not be included into the calculation of the ΔOD_{280}/min.

The enzyme sample should be diluted with citrate-phosphate buffer if $\Delta OD_{280}/\text{min} > 1.1$.

For every measurement a blank value must be recorded to determine the extent of the chemical decay of mandelonitrile, which always occurs at pH > 3, which has to be subtracted. Therefore, 950 µl of the McIlvaine–citrate–phosphate buffer are mixed with 50 µl of mandelonitrile stock solution (600 mmol/L mandelonitrile in diisopropylether) and incubated for 5 min at 25°C before the increase of the absorbance at 280 nm is determined as described for the enzyme samples.

Synthesis reaction (3-phenoxy-benzaldehyde cyanohydrin)

The corresponding buffers were saturated with 3-phenoxy-benzaldehyde. Nine hundred micro liter of the saturated buffer, 50-µL hydrogen cyanide solution (0.2 mol/L) and 50 µl enzyme solution were combined and the synthesis reaction was monitored at 307 nm in quartz cuvettes. The UV/Vis-spectra of 3-phenoxy-benzaldehyde and 3-phenoxy-benzaldehyde cyanohydrin are available in the 'supporting information' section.

Due to the very low 3-phenoxy-benzaldehyde concentration the reaction is very fast and initial rates are not available via GC-measurements or photometric assays. Therefore the reaction was monitored until the thermodynamic equilibrium was reached (if feasible). The reaction rate constant was calculated directly from the time course of the extinction by non-linear regression (Eq. 1), because the extinction correlates directly with the 3-phenoxy-benzaldehyde concentration. The non-enzymatic reaction was subtracted.

$$E = E(x) + E(t = 0) \cdot e^{(-t \cdot k)} \quad (1)$$

E Extinction (equivalent to the 3-phenoxy-benzaldehyde concentration)
$E(x)$ background absorption from the enzyme solution
$E(t = 0)$ extinction at $t = 0$s (equivalent to the 3-phenoxy-benzaldehyde concentration at the beginning of the reaction
t reaction time
k reaction rate constant.

Noteworthy, due to the large excess of hydrogen cyanide the reaction turns into a pseudo first order reaction, whereas the cyanohydrin formation from hydrogen cyanide and a carbonyl compound is usually a second order reaction. The reaction rate constant can be used directly as an equivalent for initial rate experiments. The reaction rate constant of pH 5 was set to 100% to afford a comparison with the other enzyme assays.

It has to be noticed that this activity assay was only developed to determine the enzymatic activities at elevated pH-values and in contrast to the cleavage assays a totally different technique was used. Therefore no discrete enzymatic activities were ascertainable.

HCN formation

The required amount of HCN was freshly distilled in a well-ventilated fume hood.

General procedure: 4 g of sodium cyanide were dissolved in 10 mL de-ionized water and 10 mL of 5 mol/L sulfuric acid were added drop wise within 2 min. Afterwards the combined solutions were heated up to 75 °C and formed HCN was trapped and stored at 5 °C. For the removal of water traces a spatula tip of sodium sulfate was added. All waste solutions were collected and disposed.

An electrochemical HCN-detector (Micro III G203, GfG-Gesellschaft für Gerätebau mbH, Dortmund, Germany) was placed in the fume hood for continuous monitoring.

General experimental setup

A biphasic system consisting of diisopropylether and the corresponding buffer (diisopropylether: 50% (v/v)) was applied. The substrate concentrations were 50 mmol/L 3-phenoxy-benzaldehyde and 250 mmol/L hydrogen cyanide. The reactions were carried out at 10 °C in 8 ml vials, which were shaken horizontally. Samples (50 µL) were taken from the organic phase and were added to a mixture of 700 µL dichloromethane, 50 µL trifluoroacetic anhydride and 50 µL pyridine for the acetylation procedure.

Synthesis of 2 mL of enantiopure (*S*)-3-phenoxy-benzaldehyde cyanohydrin

7.5 mL diisopropylether, 12 mL phosphate buffer pH = 7, 2 mL 3-phenoxy-benzaldehyde, 2.9 mL hydrogen cyanide and 0.42 mL enzyme solution (2,600 U) were shaken at

10 °C. Afterwards the mixture was centrifuged (at 10 °C) for phase separation. The aqueous phase was extracted twice with diisopropylether and the combined organic phases were dried with sodium sulfate. The (*S*)-3-phenoxy-benzaldehyde cyanohydrin was obtained by removal of the diisopropylether (purity > 95%, e.e.(*S*) = 97%). No further purification step was required.

Noteworthy, for the synthesis of 2 mL enantiopure (*S*)-3-phenoxy-benzaldehydecyanohydrin the substrate and enzyme concentrations were altered. Thus the ratio enzymatic/non-enzymatic reaction differ from the other results (e.g. Fig. 5). By using this improved reaction conditions, the enantiomeric excess of the product was enhanced to 97% e.e.(*S*).

GC analysis for the determination of conversion and enantiomeric excess

The conversion of 3-phenoxy-benzaldehyde to 3-phenoxy-benzaldehyde cyanohydrin (as trifluoroacetyl-derivatives) as well as the enantiomeric excesses were determined by gas chromatographic analysis with a Chiraldex Capillary Gas Chromatography Column (G-PN-γ-Cyclodextrin, Propionyl) by astec using a CP3800 gas chromatograph by Varian with a flame ionization detector (FID). Carrier gas was helium with 4 mL/min. The column temperature was constant at 130 °C. The injector- and detector-temperature were set to 250 °C.

Results and discussion

The conversion of 3-phenoxy-benzaldehyde to (*S*)-3-phenoxy-benzaldehyde cyanohydrin was chosen to demonstrate the utilization of high pH-value (pH > 6) in enzymatic cyanohydrin formation (Fig. 3).

The two-phase system diisopropylether-buffer was investigated, because of the easy experimental setup. This approach has several advantages:

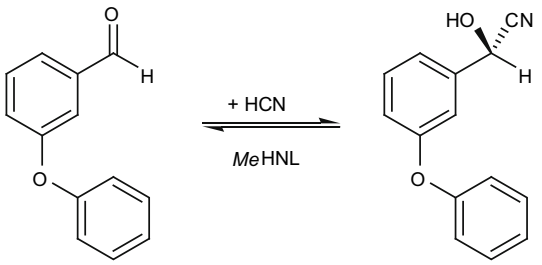

Fig. 3 Enzymatic 3-phenoxy-benzaldehyde cyanohydrin synthesis

(a) The partition coefficients of the substrates and products results in an enrichment of the reactants in the organic phase, which facilitates the product isolation.
(b) In case of a higher partition coefficient for the products an in situ product removal occurs, which potentially leads to higher grades of conversion.
(c) The easy removal of the organic phase enables an easy recycling of the enzyme within the aqueous phase.
(d) The low substrate and product concentrations in the aqueous phase avoid substrate and product inhibitions, which may positively influence the enzymatic activity.
(e) Finally, the non-enzymatic reaction is directly correlated with the substrate concentration within the aqueous phase. Due to the (usually) very high partition coefficients the substrate concentration is kept low, thereby suppressing the non-enzymatic side reaction.

Disadvantageously, in case of substrate concentrations (in the aqueous phase) below the Michaelis–Menten constant (K_M) the enzymatic reaction could be reduced. Additionally, it was found, that substrates with an unfavorable equilibrium position can be converted in organic solvent free-systems [17].

Enzyme stability

As pointed out above, the suppression of the non-enzymatic reaction is usually facilitated by a decrease of the pH-value in the aqueous phase [19, 22, 25]. In contrast, low pH-values also influence the enzyme stability of the *Me*HNL negatively (Table 1). A decrease of the pH-value to pH 3 results in a half-life time of 47 h whereas the stability increases with increasing pH (Table 1), which is

Table 1 Stability of *Me*HNL in the biphasic system diisopropylether/buffer

pH	Enzyme stability (half-life time)/h
3	47
4	84
5	>100
6	>100
7	>100
8	>100

Conditions: biphasic system diisopropylether-buffer/ratio 1:1, 10 °C, buffer concentration: 50 mmol/L, determination via the standard cleavage assay with mandelonitrile. Buffer: citrate buffer (pH = 3–5), citrate phosphate buffer (pH = 6–7) and Tris–buffer (pH 8)

even enough for poorly accepted substrates. Noteworthy, the addition of the substrates (carbonyl compound and hydrogen cyanide) also decreases the enzyme stability significantly [17]. Thus, the decrease of pH in the biotransformation makes the application of higher amounts of enzyme necessary.

Enzyme activity

On the other hand, the variation of the pH-value also influences the enzyme activity. Therefore, several enzyme assays are available to determine the enzyme activity of hydroxynitrile lyases at different pH-values. Usually the enzymatic cleavage of mandelonitrile is followed, whereas generally the racemic compound is applied. To obtain the enzyme activity, the non-enzymatic cleavage reaction is subtracted. Other enzyme assays use acetone cyanohydrin (and other substrates), whereas the conversion is measured with a spectrometric HCN-assay [26, 27]. Due to the very fast non-enzymatic background reaction enzyme activities at pH 5 and above are hardly measurable.

In summary, these enzyme assays are following the cleavage reaction, whereas the results are usually used for the discussion of the synthesis reaction (e.g. enzyme activity). In contrast to this, we want to describe a spectrometric assay, which can be used also at high pH-values for the synthesis reaction to obtain reliable results.

In contrast to the cleavage reaction assays the synthesis of 3-phenoxy-benzaldehyde cyanohydrin was monitored spectrometrically until the reaction equilibrium was reached. The reaction rate constant was calculated and to evaluate the enzyme activity at different pH-values (see also experimental section) (Fig. 4).

As demonstrated in Fig. 4 the pH-optima determined for the cleavage and synthesis reaction differ significantly. Both the cleavage of mandelonitrile and acetone cyanohydrin [28] showed a pH-optimum around pH 5.5, while a pH-optimum of pH > 8 was found for the synthesis of 3-phenoxy-benzaldehyde cyanohydrin. Beyond pH 8 the non-enzymatic side-reaction overlaps again significantly the enzymatic reaction, in accordance with the cleavage assays.

3-Phenoxy-benzaldehyde cyanohydrin synthesis at elevated pH-values

Finally, the high enzyme activity and stability at elevated pH-values and the low non-enzymatic reaction of 3-phenoxy-benzaldehyde permit the application of the cyanohydrin synthesis in a two phase system, because of the high ratio of enzymatic to non-enzymatic reaction. This

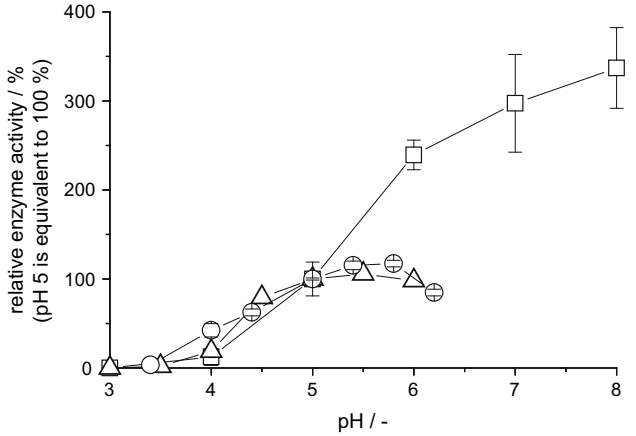

Fig. 4 Enzymatic activity in dependence of pH-value. *Circle* cleavage reaction assay with mandelonitrile; McIlvaine–citrate–phosphate-buffer (range: pH = 3.4–6.2). *Triangle* cleavage reaction assay with acetone cyanohydrin. *Square* synthesis reaction assay with 3-phenoxy-benzaldehyde cyanohydrin; 25 °C, 50 mmol/L citrate buffer (pH = 3–5), 50 mmol/L citrate phosphate buffer (pH = 6–7), 50 mmol/L Tris–buffer (pH 8). The results were scaled to 100% for pH 5, because the different reaction systems prevent a useful comparison. Unfortunately, because of the very fast non-enzymatic reaction no reliable data above pH = 8 was available

was also proven with substrate concentrations up to 500 mmol/L.

For the investigation of the synthesis in the spectroscopic assay using a substrate saturated aqueous solution the concentration and therefore the volumetric productivity is limited. To develop a suitable protocol for production purposes the observed pH influence was investigated in a two-phase system as well (Fig. 5). As within the spectroscopic assay the conversion is increasing with the pH. Surprisingly, the pH remains high up to pH 8, but is higher at somewhat lower pH.

While conversion is rather low at pH 4, the velocity of the reaction increases significantly from pH 5 to pH 6 yielding very good e.e., whereas more than 22 h are needed to obtain good conversions. The pH-optimum for this reaction was found near pH 7. Interestingly, even at pH 8 still an enantioselective reaction with e.e.(S) = 85% was possible. Beyond pH 8 the non-enzymatic racemic reaction overlaps the enzymatic reaction and yields in a decrease of the enantiomeric excess. However, even at pH 9 a small enantiomeric excess was measurable, demonstrating the potential of this synthetic concept.

Comparison with the variant *Me*HNL–W128A

Finally, the wild type *Me*HNL and the variant *Me*HNL–W128A were compared concerning the synthesis of 3-phenoxy-benzaldehyde cyanohydrin. As demonstrated in Fig. 6 the MeHNL-variant is significantly faster then the

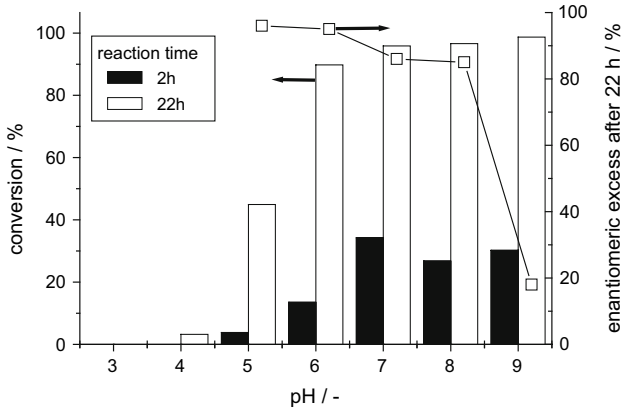

Fig. 5 Dependence of conversion and enantiomeric excess from the pH-value within a biphasic reaction medium. Reaction conditions: 10 °C, 50 mmol/L buffer, biphasic reaction system diisopropylether–buffer 1:1, 50 mmol/L 3-phenoxy-benzaldehyde, 250 mmol/L hydrogen cyanide, 20 U/mL *Me*HNL buffer: citrate buffer (pH = 3–5), citrate phosphate buffer (pH = 6–7) and Tris–buffer (pH 8, 9)

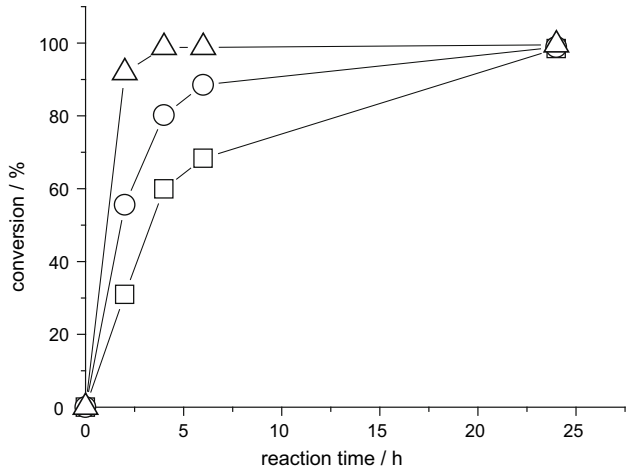

Fig. 6 3-Phenoxy-benzaldehyde cyanohydrin formation with *Me*HNL (wildtype and the variant *Me*HNL–W128A) reaction conditions: 10 °C, 50 mmol/L citrate phosphate buffer pH = 7.0, biphasic reaction system diisopropylether-buffer 1:1, 50 mmol/L 3-phenoxy-benzaldehyde, 250 mmol/L hydrogen cyanide. *Triangle* 20 U/mL *Me*HNL-W128A (e.e.(*S*) after 24 h: 84%). *Circle* 50 U/mL *Me*HNL-wild type (e.e.(*S*) after 24 h: 85%). *Square* 20 U/mL *Me*HNL-wild type (e.e.(*S*) after 24 h: 75%)

wild type, even at lower enzyme concentrations, which is consistent with the preferred acceptance of bulky substrates by this variant [15].

With 20 U/mL *Me*HNL full conversion was reached after 6 h, whereas it took nearly 24 h to obtain full conversion with the wild type enzyme, which was additionally connected with a decrease of the enantiomeric excess to 75% e.e.(*S*). This demonstrates again the dependence of the enantiomeric excess from the ratio of the enzymatic/non-enzymatic reaction velocities. By increasing the enzyme concentration up to 50 U/mL the enantiomeric excess increases to 85% and even higher enzyme concentrations to 97 e.e.(*S*), which is the maximum for this enzyme.

When using only 20 U/mL of the variant an e.e. of 84%(*S*) could be found. The e.e. for the (*S*)-enantiomer can be increased to 90% with higher enzyme loads.

Conclusion

The formation of cyanohydrins catalyzed by hydroxynitrile lyases was investigated at unusually high pH values. We pointed out, that the ratio enzymatic/non-enzymatic reaction is decisive for the enantioselective reaction. Although these high pH-values are only applicable with substrates showing a slow non-enzymatic reaction, the dramatic increase of enzymatic activity at elevated pH-values enables the conversion of substrates, which show only low yields in the standard reaction systems. Under these circumstances even unfavorable substrates can be converted.

Finally, the potential of the "high-pH-approach" was presented by the synthesis of 2 mL enantiopure (*S*)-3-phenoxy-benzaldehyde cyanohydrin [purity > 95%, 97% e.e.(*S*)], using wild type *Me*HNL.

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Publikation 5

“Enzyme catalysis in non-aqueous media – past-present-future“

Dreyer, S.; Lembrecht, J.; **Schumacher, J.**, Kragl, U.
in R. Patel (Editor) *“Biocatalysis in the Pharmaceutical and Biotechnology Industries”*, CRC-Press, Taylor & Francis Group, Boca Raton, pp 791-828 (2006)

Anteile: Dreyer, S. (30%); Lembrecht, J. (30%); **Schumacher, J. (30%)**; Kragl, U. (10%)

Einleitung zu Publikation 5

Der Buchbeitrag *„Enzyme catalysis in non-aqueous media – past-present-future“* in R. Patel (Editor) *“Biocatalysis in the Pharmaceutical and Biotechnology Industries”* präsentiert als Publikation 5 die Verwendung von organischen Lösungsmitteln in der Biokatalyse (Abbildung 8-5).

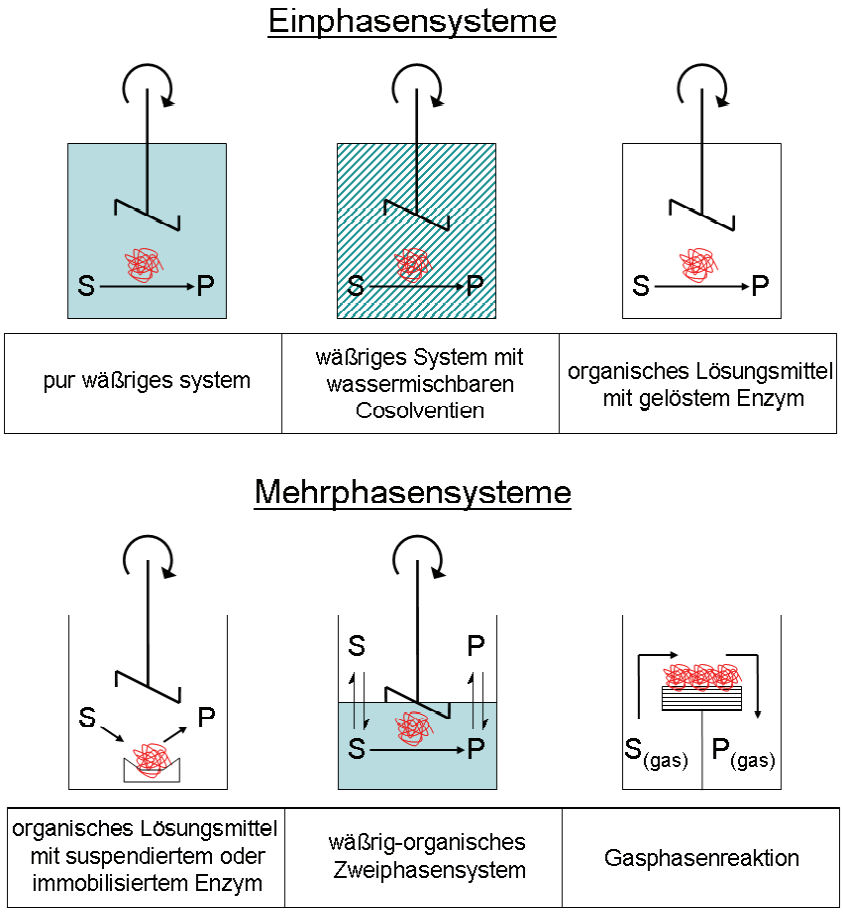

Abbildung 8-5: Vorgehensweise Publikation 5

Die einzelnen Enzymklassen werden an dieser Stelle systematisch entsprechend ihre Verwendung in nicht-wässrigen Medien hin dargestellt. Es zeigte sich dabei, dass überwiegend die Oxidoreduktasen, Hydrolasen und Lyasen mit organischen Lösungsmitteln eingesetzt werden. Dies korreliert mit der Anwendung dieser Enzymklassen in zahlreichen Synthesebeispielen, wobei üblicherweise schwerlösliche nicht-natürliche Substrate umgesetzt werden.

33 Enzyme Catalysis in Nonaqueous Media: Past, Present, and Future

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33.1 INTRODUCTION

Nature has designed its biocatalysts to perform best in an aqueous surrounding, neutral pH, and temperatures below 50°C. However, these conditions are often contrary to the requirements of the process engineer or chemist to optimize a reaction with respect to volumetric productivity or an easy downstream processing when substrates and/or products are not easily soluble in water. To overcome these bottlenecks addition of organic solvents is common practice. This has been used by various authors over the years, but a breakthrough was achieved during the second half of the 20th century [1–3].

The solvent can either be water-miscible or water-immiscible, resulting in one- or two-phase systems. Depending on the solvent phase the enzyme will be dissolved or only suspended. Thus, the systems might be liquid, liquid–liquid, or liquid–solid, but even gas-phase reactions are gaining increasing attention. The possibilities are summarized in Figure 33.1. The aim of this chapter is to highlight some of the developments over the years. Thus, in no way can it be exhaustive.

In a pure aqueous system the solvents implied are the dissolved enzyme(s), cofactors and cosubstrates if needed, the substrate, and the product. In a pure organic phase most often the enzyme is not soluble and therefore applied in suspension either in its native form or on a carrier. In a two-phase system formed by water and an organic solvent the enzyme is either dissolved in the bulk aqueous phase or can be localized at the interface. The organic phase then can be seen as a reservoir for a poorly water-soluble substrate. During the reaction the substrate and the product formed will partition between the two phases. On the one hand, using two-phase systems for enzyme-catalyzed reactions might have several advantages. Firstly, higher concentrations of hydrophobic substrates and therefore higher volumetric productivities can be achieved. Secondly, no enzyme inhibition by high substrate concentrations will occur. Thirdly, an easy workup by phase separation is possible. Fourthly, "free immobilization" of the enzyme and cofactor in the aqueous phase allows reuse of the enzyme and the cofactor [4–6].

On the other hand, the influence of the organic phase on enzyme activity, stability, and regio- and stereospecificity has to be evaluated. The choice of the organic solvent is mainly influenced by the enzyme behavior and by the partitioning behavior of substrate and product [7–9]. It should be noted that despite all success there is no general rule as to which solvent is "enzyme friendly." To a certain extent, the log *P* concept, based on the distribution

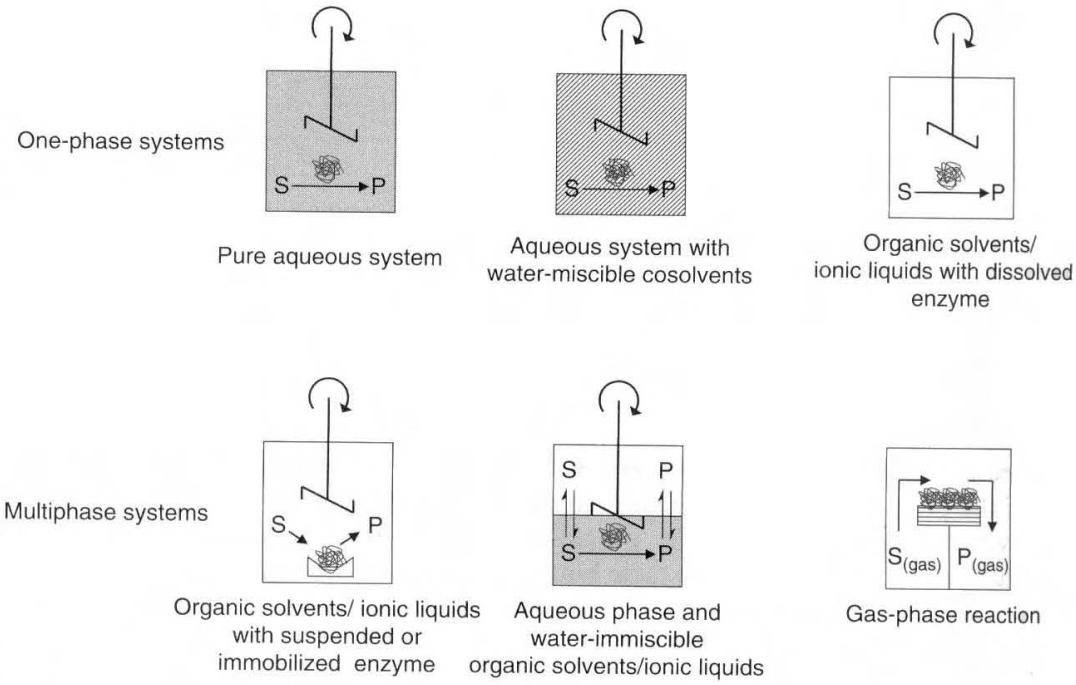


FIGURE 33.1 Possible reaction system for biocatalysis in nonconventional media.

coefficient between water and *n*-octanol, can be used as a guideline [10]. In general, solvents with a log $P > 3$ such as xylene (3.1) or hexane (3.9) are less deactivating than those with a low log P such as ethanol (−0.24). Surprisingly, *tert*-butanol (0.35) stabilizes enzymes [11]. Certainly the hydrophilicity of the cosolvent is important, as it allows interaction and breaking of hydrogen bonds that are stabilizing the tertiary structure of the enzyme. However, not only have common organic solvents been used for biocatalysis, but supercritical CO_2 [12] and recently even ionic liquids (ILs) have also been shown to be compatible with enzymes or whole cells [13–16].

33.2 EC 1: OXIDOREDUCTASES

The oxidoreductases represent the first group of the classification formulated by the enzyme community (EC numbers). Enzymes that are part of this group reversely catalyze the oxidation and reduction of substrates by transferring two electrons. Mostly they need a cofactor, also called coenzyme, which activates the enzyme and acts as a mediator between the active site of the biocatalyst and the substrate [17]. These cofactors are small organic nonprotein molecules that can covalently or noncovalently bind to the inactive apoenzyme to build the active catalyst (haloenzyme). Such coenzymes are molecules like nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and flavine adenine dinucleotide (FAD). As can be seen in Table 33.1, oxidoreductases are divided into 22 subclasses.

TABLE 33.1
Subclasses of the Oxidoreductases

EC 1 Oxidoreductases	
EC 1.1	Acting on the CH–OH group of donors
EC 1.2	Acting on the aldehyde or oxo group of donors
EC 1.3	Acting on the CH–CH group of donors
EC 1.4	Acting on the CH–NH_2 group of donors
EC 1.5	Acting on the CH–NH group of donors
EC 1.6	Acting on the NADH or NADPH
EC 1.7	Acting on the other nitrogenous compounds of donors
EC 1.8	Acting on a sulfur group of donors
EC 1.9	Acting on a heme group of donors
EC 1.10	Acting on diphenols and related substances of donors
EC 1.11	Acting on peroxide as acceptor
EC 1.12	Acting on hydrogen of donors
EC 1.13	Acting on single donors with incorporation of molecular oxygen (oxygenases)
EC 1.14	Acting on paired donors with incorporation or reduction of molecular oxygen
EC 1.15	Acting on superoxide as acceptor
EC 1.16	Oxidizing metal ions
EC 1.17	Acting on the CH or CH_2 groups
EC 1.18	Acting on iron–sulfur proteins as donor
EC 1.19	Acting on reduced flavodoxin as donor
EC 1.20	Acting on phosphorus or arsenic in donor
EC 1.21	Acting on X–H and Y–H to form an X–Y bond
EC 1.97	Other oxidoreductases

33.2.1 Alcohol Dehydrogenases

The biggest subclass of the oxidoreductases comprise alcohol dehydrogenases (ADHs; EC 1.1). In the past, the ADHs were typically used in a buffered aqueous system because they and their cofactors are sensitive against organic solvents. For example, Hummel published the reduction of acetophenone to (*R*)-1-phenylethanol by the ADH from *Lactobacillus kefir* in potassium phosphate buffer with NADPH regenerated by a glucose-6-phosphate dehydrogenase (G6PDH) [18].

An oft-applied enzyme is the yeast alcohol dehydrogenase (YADH). The development started with the use of whole cells as biocatalysts to avoid the cofactor regeneration in an additional reaction step. Since the end of the 20th century YADH has also been deployed in nonconventional media. Howarth et al. reported that it is possible to use immobilized baker's yeast as whole cells in ILs mixed with water (10:1) to reduce prochiral ketones [19,20]. Organic solvents offer the chance to selectively form one of the enantiomers. It has been reported that both enantiomers were produced when reducing 2-oxohexanolate by baker's yeast in water, but when the biotransformation was conducted in benzene, the (*R*)-alcohol was formed in high yields [21]. Although the ADH can be successfully used under nonconventional conditions, it should be noted that the catalytic activity strongly depends on the water activity in the system [22].

Further, Liao et al. performed a YADH covalently bonded onto Fe_3O_4 magnetic nanoparticles, which is active in a water/AOT/isooctane microemulsion (Figure 33.2). The residual activity immobilized YADH after 700 h was 78% and, in contrast to that, the free YADH loses most of its activity in the same reaction system after 1 h [23]. Beyond this, YADH is highly stable and active in reverse micelles of AOT/isooctane depending on water content, pH, and time [24,25].

For further research cross-linked enzyme aggregates (CLEAs), gas-phase reactions, and combinations of different organic solvents would be a promising field. However, there are still some open questions in the areas of one- and two-phase systems that have to be analyzed for a better understanding of the mechanism.

Since the last 10 to 15 years there have appeared a number of reports about ADHs in nonconventional media. A new ADH from *L. brevis* (LBADH), first mentioned by Riebel,

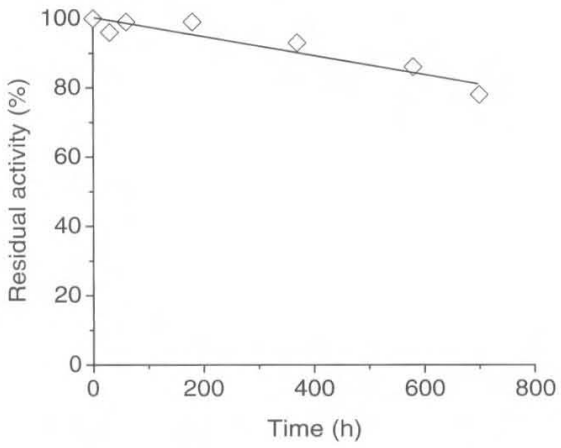


FIGURE 33.2 Storage stability of bound YADH at 25°C. The activity measurement was preformed in 10 ml microemulsion solution at 0.1 M AOT, 0.2 mM NADH, 0.1 M 2-butanone, 25°C, and $\omega_0 = 25$. The concentration of bound YADH was 0.5 mg/ml. The initial 100% absolute values of activity for bound YADH was 8 nmol/(min·mg). (From Liao, M.H. and Chen, D.H., *J. Mol. Catal. B Enzym.*, 18, 81–87, 2002.)

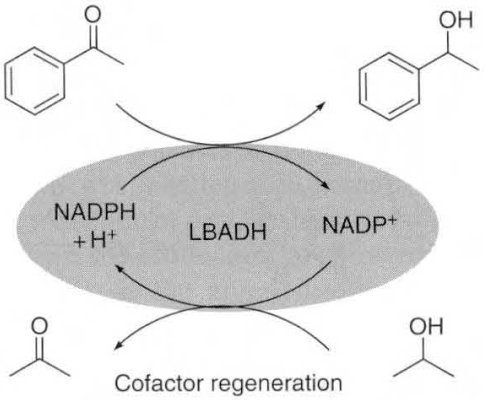


FIGURE 33.3 Model reaction in the gas phase: reduction of acetophenone with cofactor regeneration by 2-propanol oxidation. Enzyme and cofactor are immobilized on glass beads. (From Ferloni, C. et al., *Biokatalyse* (Transkript Sonderheft), 105–108, 2003.)

is able to catalyze the reduction of prochiral ketones [26]. This can be done not only in buffer but also in many different reaction systems. The advantage of all these systems is a higher stability of the LBADH. As published by Filho et al. the half-lives of ADHs are not directly related to the log *P* values of the solvent, but it is possible to appraise the miscibility with water and thus enable the contact between the enzyme and the organic solvent [27].

Ferloni et al. reported that the immobilized LBADH shows an enzyme activity of 100% for the reduction of substrates in the aqueous phase and also in the gas phase (Figure 33.3) [28]. This implies a high reactivity of the ADH to the reduction of acetophenone in the gas phase. The conversion of the substrate and the reactivity of the enzyme depend on relative moisture in the reaction system, on the pressure, and on the molecular ratio of the cosubstrate 2-propanol to the substrate.

The reduction of prochiral ketons catalyzed by LBADH including substrate-coupled regeneration with 2-propanol has been used for the production of chiral alcohol on a 10 to 100 kg scale, e.g., ethyl-(*R*)-3-hydroxybutyrate or (*R*)-2-octanol. The reaction was performed in a one-phase system with an extraction step at the end [29]. Schumacher et al. researched the LBADH-catalyzed reduction of short aliphatic ketones in a one-phase system with cosolvents [30]. They investigated an increasing enantiomeric excess (ee) of (*R*)-butan-2-ol (36.5 to 43.0%) by increasing the amount of acetonitrile from 0% (v/v) to 24.5% (v/v).

It is also possible to arrange the reaction in a two-phase system (Figure 33.4). Eckstein et al. investigated the reduction of 2-octanone to (*R*)-2-octanol with the cofactor regeneration by the same enzyme in two different binary systems [31]. In the first system of methyl *tert*-butyl ether (MTBE) and buffer the reduction catalyzed by the free LBADH achieved a conversion of 61% after 180 min. In contrast, the reaction is much faster when using an IL instead of an organic solvent as second phase. In the case of [BMIM][$(CF_3SO_2)_2N$]/buffer the conversion after 180 min reaches 88%.

Using the LBADH in a two-phase system has two advantages. Firstly, the concentration of poorly water-soluble substrates can be raised in the total system for even decoupling as reported by Kroutil et al. [32]. Secondly, there is an increase of stability of the LBADH in comparison to conversion in one-phase systems with cosolvents. It depends on the organic solvent in comparison to the conversion in buffer [27]. The half-life of the LBADH is effected by the nature of the organic solvent that is used as second phase, although the contact between the enzyme and the organic media should be marginal (Figure 33.5) [33].

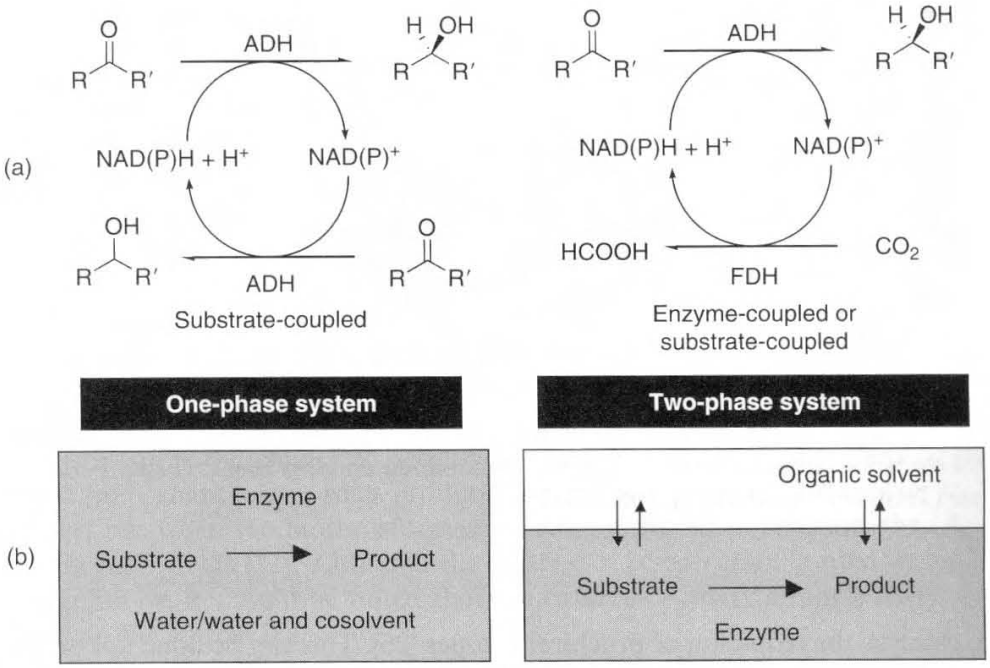


FIGURE 33.4 (a) Cofactor regeneration with substrate-coupled and enzyme-coupled approach. (b) Scheme of a one-phase system and a two-phase system for enzyme catalysis. (From Eckstein, M. et al., *Chem. Comm.*, 1084–1085, 2004).

The enzymatic reduction by LBADH requires NADPH, an expensive cofactor. To configure this reaction economically an *in situ* regeneration is necessary. Eckstein et al. reviewed various methods for one- and two-phase systems to minimize the required cofactor concentration [29]. A common method is the use of a second substrate, mostly 2-propanol, to recycle the cofactor into its catalytically active form. This substrate-coupled regeneration is

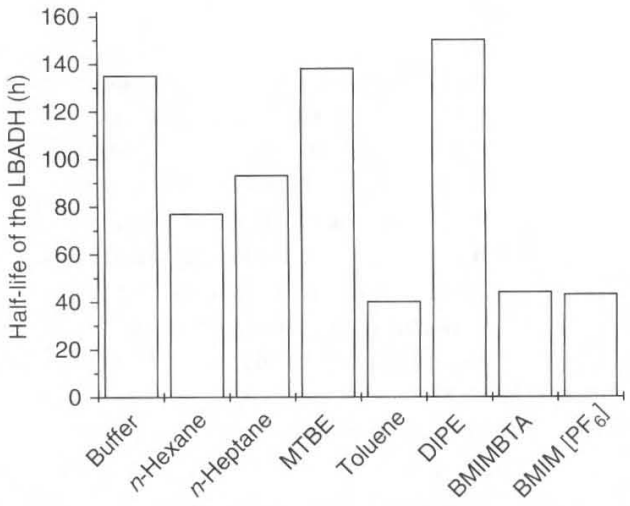


FIGURE 33.5 Half-life of the LBADH in buffer and different two-phase systems. Conditions: total volume 8 ml, organic solvent/water 1:1 (α = 1), 3 U/ml (aq) LBADH, 0,1 mM (aq) $NADP^+$/NADPH, 30°C, 900 rpm, phosphate buffer (50 mM, 1 mM $MgCl_2$, pH 7,0). (From Lembrecht, J., unpublished data, 2006.)

mostly implemented by the same enzyme as that for the reduction of the ketone. Alternatively, an enzyme-coupled regeneration can be used. In this case, a second enzyme is deployed to restore NAD(P)H. Firstly, a regeneration by formate dehydrogenase (FDH) that is able to oxidize formate to CO_2 [34] has been reported, but also an application of glucose dehydrogenase (GDH), which oxidizes D-glucose, or D-glucose-6-phosphate dehydrogenase, which converts D-glucose-6-phosphate, was mentioned in literature. These are the two mainly used regeneration methods for the reduction of prochiral ketones in one- and two-phase systems [35].

Another possibility to permit the production of chiral hydrophobic alcohols catalyzed by ADHs in nonconventional media is the immobilization on a support. De Temiño et al. reported a higher stability of ADH from *L. kefir* by entrapping the enzyme and its cofactor in polyvinyl alcohol gel beads [36]. In the case of an immobilized enzyme it is possible to convert in pure organic solvents. An encapsulation in reverse micelles also causes a higher half-life time of ADHs in pure organic solvents [25]. To create a higher stability of the LBADH the preparation of CLEAs has been mentioned by Mateo et al., although the cross-linked ADH exhibits a recovered activity of 7 to 10% relative to the native enzyme cross-linked with dextran polyaldehyde [37].

To facilitate the catalysis in pure organic solvents or in the environment-friendly ILs genetic rearrangement of the enzyme would be a possibility.

33.2.2 Laccases

Laccases are the second group of oxidoreductases that will be discussed in this chapter. These copper-containing enzymes oxidize substrates by reduction of molecular oxygen to water. Typically, laccases are deployed in textile, pulp, and paper industries, but they can be used as "green" catalysts in organic synthesis as well. Khmelnitsky studied the degeneration of laccase from *Polyporus versicolor* and other enzymes for the oxidation of a model substrate in many different organic solvents [38]. Laccases, which are very sensitive to nonconventional conditions, are only active in organic solvents till a limiting concentration of the cosolvent is exceeded. This barrier depends on the enzyme and also on the cosolvent; so the border where the enzyme is still native (catalytically active) must be determined for each protein.

Commonly, laccases are used in buffer solution, e.g., in the oxidative polymerization of 4-chloroguaiacol [39]. It has been found that a pH between 4 and 5 is the optimized condition for the described reaction.

As a result of the inactivation by organic solvents immobilization is a typical method for the laccase-catalyzed synthesis in organic solvents. Pilz et al. published the synthesis of coupling products of phenolic substrates (Figure 33.6) in different reactors [40]. They used

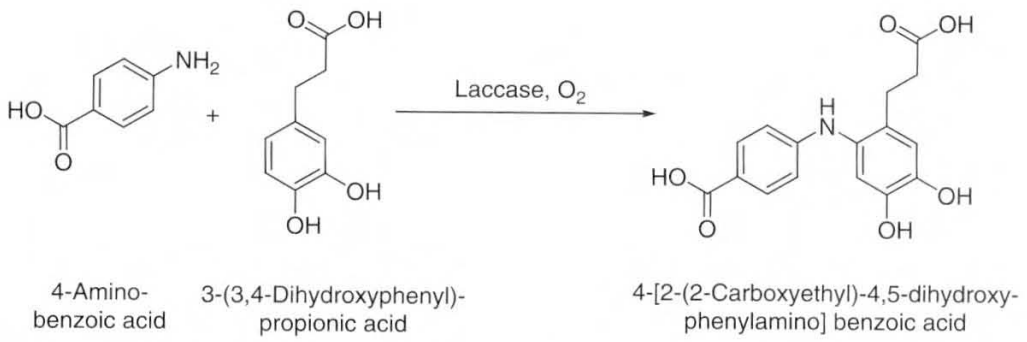


FIGURE 33.6 Cross-coupling reaction catalyzed by laccase from *Pycnoporus cinnabarinus*. (From Pilz, R. et al., *Appl. Microbiol. Biotechnol.*, 60, 708–712, 2003.)

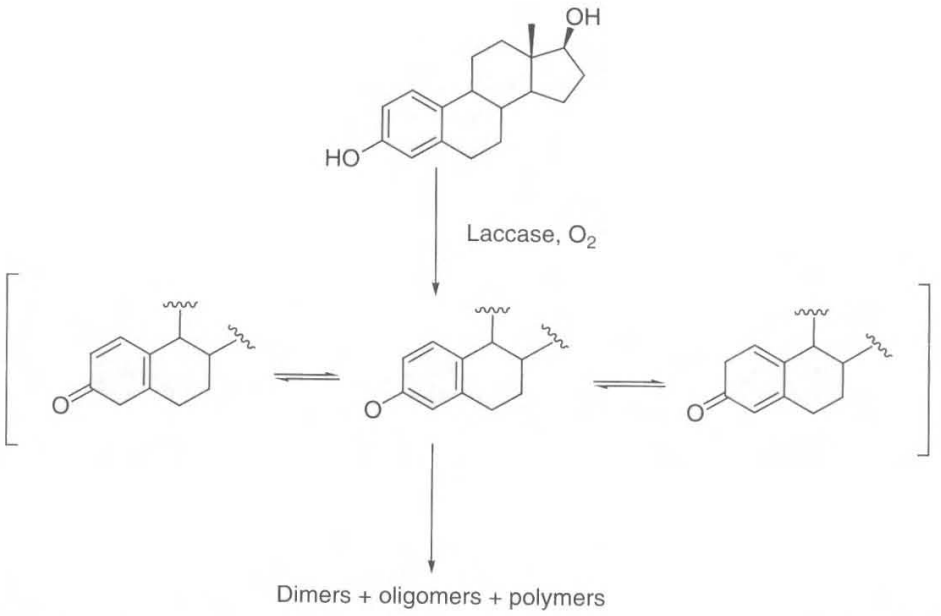


FIGURE 33.7 Laccase-mediated oxidation of β-estradiol. (From Nicotra, S. et al., *Tetrahedron Asymmetry*, 15, 2927–2931, 2004.)

an immobilized laccase prepared from the white rot fungus *Pycnoporus cinnabarinus* in a stirred tank reactor (STR) and also in a continuously operated enzyme-membrane reactor (EMR) in acetate buffer (pH 5). Immobilization of the enzyme on a support offers the possibility to reduce the amount of enzyme required for the reaction.

It has also been reported that the oxidation of the steroid hormone 17β-estradiol by laccase from *Mycelypthora* and *Trametes pubescens* in organic media is feasible. Nicotra et al. accomplished the laccase-mediated oxidation in pure organic solvents and also in two-phase systems using the enzyme immobilized on glass beads (Figure 33.7) [41]. As pure solvent a mixture of dioxin and water-saturated toluene has been applied and as two-phase system AcOEt/buffer (pH 4.5) was utilized. The oxidation of β-estradiol by laccase from *P. versicolor* was described more than 30 years ago in one of the first papers on the use of enzymes in two-phase systems [42].

In the future it might be possible to use laccases not only in the paper industry but also to produce new polymers that cannot be synthesized by organometallic catalysts or other enzymes. Therefore, a further development of immobilization methods to generate highly active and stabile laccases in organic media is necessary. Perhaps it would be possible to deploy immobilized laccases in a gas-phase reaction.

33.2.3 Monooxygenases

The third subclass of oxidoreductases that will be discussed is made up of monooxygenases. This subclass of the EC 1 catalyzes the oxidation of unsaturated substrates to oxiranes or lactones by reducing molecular oxygen. Monooxygenases are dependent on cofactors, mostly NADH and NADPH, so around 10 years ago the deployment of whole cells as biocatalyst was common. Schmid et al. availed whole cells containing styrene monooxygenase recombinant in *Escherichia coli*. [43]. The synthesis of (*S*)-styrenes from styrene and its derivates was arranged in a two-phase system containing buffer and dioctyl phthalate. Because of the

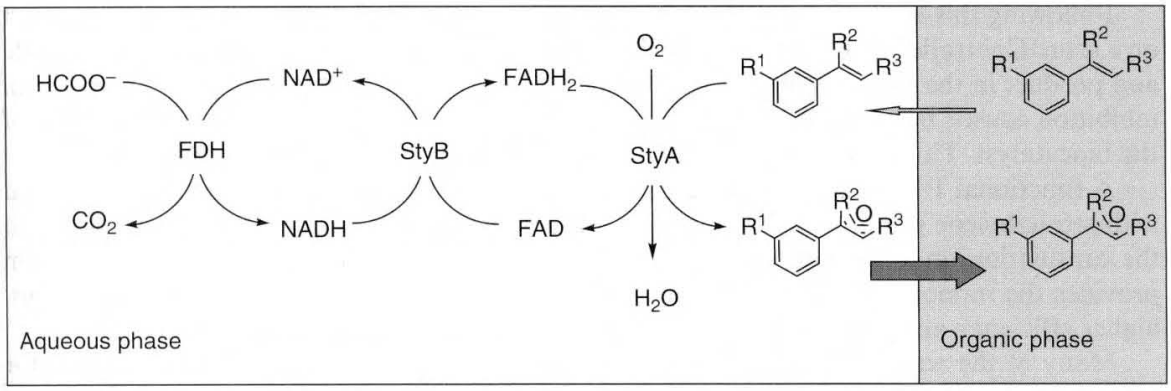


FIGURE 33.8 Reaction pathway during biocatalytic epoxidation in a two-phase system. The organic phase serves as a substrate reservoir and product sink. In the aqueous phase, formate dehydrogenase (FDH) and formate were used for regeneration of NADH. StyB transfers the reducing equivalents from NADH to flavine adenine dinucleotide (FAD). $FADH_2$ and oxygen are cosubstrates for olefin epoxidation by StyA. R^1 = H, Cl; $R^2 = R^3$ = H, CH_3. (From Hofstetter, K. et al., *Angew. Chem. Int. Ed.*, 43, 2163–2166, 2004.)

toxicity of the substrates the second phase could work as a reservoir to keep the substrate concentration low in the environment of the enzyme. The two-phase system also allows an *in situ* product removal (IPS) to isolate the toxic and water-labile product.

A biocatalytic asymmetric epoxidation with NADH regeneration in organic–aqueous emulsions has also been published [44]. As can be seen in Figure 33.8 there are two styrene monooxygenases involved, StyA and StrB (flavin- and NADH-dependent). As reported by Schmid et al. the second phase acts as reservoir of the substrate and also as an *in situ* extraction of the product [43]. The yield and also the ee of the epoxidation are comparable to the epoxidation by whole cells.

Alternatively, monooxygenases can transform racemic bicyclo[3.2.0]hept-2-en-6-one to chiral lactones and thioanisole (methyl phenyl sulphide) into its chiral (*R*)-sulphoxide [45,46]. In this case, coimmobilization of the enzyme and the cofactor is necessary to keep the biocatalyst active in the organic reaction media, e.g. on Eupergit C [45]. However, it is also possible to use a membrane reactor for removing the product continuously from the reaction media. Hilker et al. reported an *in situ* substrate feeding/product removal for the Baeyer–Villiger oxidation process catalyzed by cyclohexanone monooxygenase (CHMO) (Figure 33.9) [46].

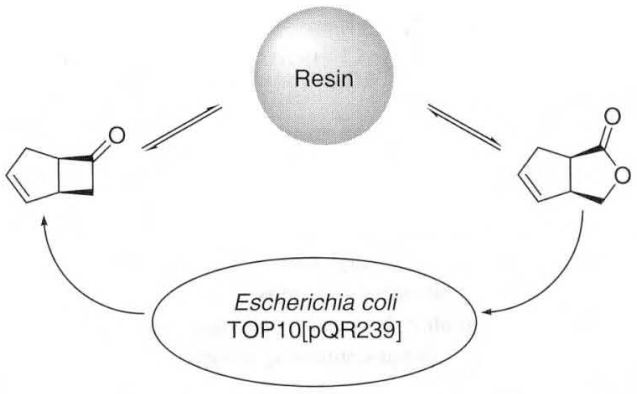


FIGURE 33.9 Regiodivergent Baeyer–Villiger oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one. (From Hilker, I. et al., *Org. Lett.*, 6, 1955–1958, 2004.)

Following this methodology they adsorbed substrate and, after reaction, also the product on a resin. Controlled by the adsorption/desorption equilibrium, the concentration of substrate and product in the aqueous bulk phase, containing enzyme and cofactor, is low. Thereby an inhibition caused by either the substrate or the product is minimized without stabilization of the biocatalyst. This approach has been first described by Zmijewsky et al. [47,48].

A functional P450cam monooxygenase was created in water–oil (w/o) emulsion formed with tetraethylene glycol dodecyl ether as a surfactant [49]. This can be used alternatively to the capsulation on a support, because the inner aqueous compartment of the w/o emulsion provides the monooxygenase with a cell-like environment in the organic bulk phase. Thus, higher efficiency and productivity of the biocatalyst can be achieved.

Many of the reaction systems that are possible (Figure 33.1) have not been reported for monooxygenases as yet. It might be effective to study the conversion of different substrates in the gas phase, because one of the reactants, oxygen, is gaseous.

In this chapter the possibilities of different reaction systems for the catalysis of oxidoreductases have been shown by some examples. Oxidoreductases are, in addition to the hydrolases, the most important EC group for industrial processes, because they are available in a large scale. It could be revealed in this chapter that the oxidoreductases are able to catalyze conversions of many different substrates in various reaction systems just like two-phase systems. In the future is would be interesting to have a look at CLEAs as a promising immobilization method [37]. However, it should be kept in mind that oxidoreductases are often used in whole-cell processes adding different requirements to the reaction system [168].

33.3 EC 2: TRANSFERASES

Transferases catalyze the transfers of functional residues of various substrates. They are divided into nine subclasses, shown in Table 33.2 [50].

The utility in organic synthesis for transferase-catalyzed reactions in nonconventional media is very low, compared to their catalytic importance in the living organism [51]. Nevertheless, several reports of organic solvent effects on transferases have been published in the past. An early example deals with the effect of the organic solvents ethanol and 1,4-dioxane on a citrate synthase from pig heart [52]. For both solvents it has been found

TABLE 33.2
Subclasses of the Transferases

EC 2 Transferases	
2.1	Transferring one-carbon groups
2.2	Transferring aldehyde or carbon groups
2.3	Acyltransferases
2.4	Glycosyltransferases
2.5	Transferring alkyl or aryl groups, other than methyl groups
2.6	Transferring nitrogenous groups
2.7	Transferring phosphorous-containing groups
2.8	Transferring sulphur-containing groups
2.9	Transferring selenium-containing groups

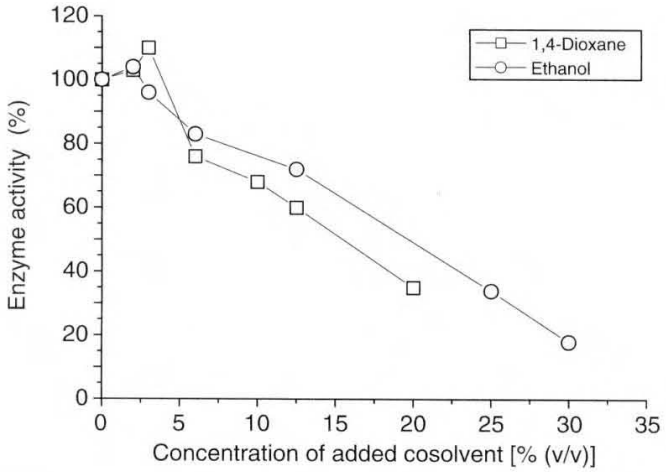


FIGURE 33.10 Effect of 1,4-dioxane and ethanol on citrate synthase from pig heart.

that with increasing amount of added solvent the enzyme activity decreases (Figure 33.10). The 50% inhibition was found to be at 16% dioxane and 19% ethanol.

In 1979 Singh and Wang reported the effects of organic solvents on a glycogen phosphorylase kinase from rabbit skeletal muscle [53]. It was observed that several organic solvents stimulate the enzyme up to a 28-fold enhancement (acetone). An overview is given in Table 33.3.

Screening the enzyme activity in correlation with increasing concentration of ethanol, a complex behavior has been observed. With an increasing amount of ethanol a relatively sharp maximum at 1.72 M was found, yielding in a kinase activity above 4 units/mg. This can be explained by ethanol-caused modification of the affinity of the protein phosphorylase b toward the kinase, which can also be done by a modification of the pH. At this point the pH-activity profile can also be modified by the organic solvent ethanol (Figure 33.10).

As shown in Figure 33.11 the activating effect is also transferable to other pH values, resulting in an activating effect. With an increase of pH the activation became negligible above a pH of 8.5; however, the causes for the behavior are not known. The authors were also able to show that all the observed effects caused by ethanol are mostly reversible, diluting out the ethanol. Therefore, the origin of the observed effects is not completely known, because no kinetic data were available.

TABLE 33.3
Enhancement of Phosphorylase Kinase–Catalyzed Synthesis Effected by Various Organic Solvents at 1 M Each (Unactivated Enzyme)

Solvent	Stimulation (-fold)
None (buffer)	1.0
Methanol	3.3
Ethanol	8.8
2-Propanol	18.6
Acetone	27.5
Dimethyl sulfoxide	17.8
Tetrahydrofuran	18.6

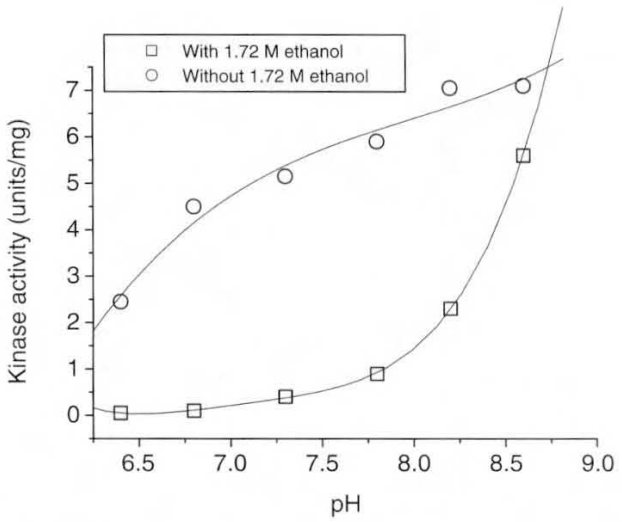


FIGURE 33.11 Ethanol-induced modification of pH dependence of (unactivated) kinase activity.

Contrarily to this, an influence of ethanol on the enzyme kinetics has been investigated for a myristoyl CoA:protein *N*-myristoyltransferase (NMT) [54]. For this transferase similar results were obtained following addition of the organic solvents, in this case for ethanol and acetonitrile (Figure 33.12).

For both added organic solvents it has been found that with increasing amount of organic solvent the NMT activity rises. After a certain amount the activity decreases again, probably because of the inactivation of the enzyme through the organic solvent.

It is noteworthy that at 10% (v/v) ethanol the NMT activity increased nearly fivefold, but the cause of this activation has yet to be found in the altered enzyme kinetic constant. This has been observed at $pp60^{scr}$ (a peptide substrate from NH_2-terminal sequences), which showed nearly a fivefold rise in the V_{max} value during an additional ethanol concentration of 10% (v/v) at a constant K_M value (Table 33.4).

It has been presumed that this activation is caused by a time-and-concentration-dependent unfolding mechanism. So an interference of the organic solvent molecules that arises from their different dielectric constants could lead to secondary effects on the counter-

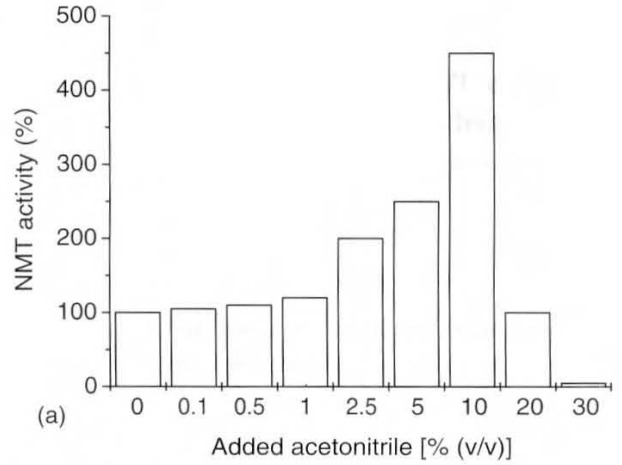


FIGURE 33.12 Effect of (a) acetonitrile and

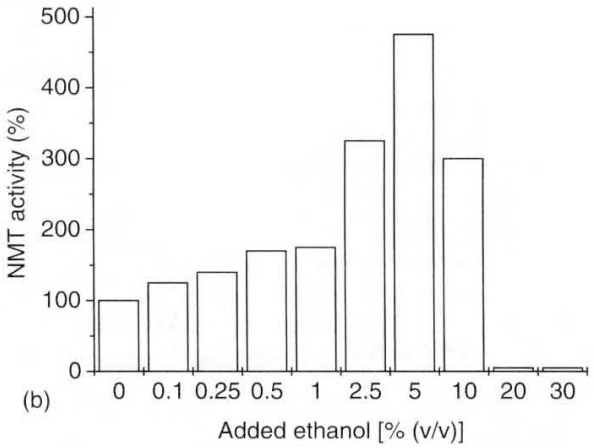


FIGURE 33.12 (continued) (b) ethanol on *N*-myristoyltransferase (NMT).

TABLE 33.4
Effect of Ethanol at Kinetic Constants

Substrate	Ethanol [% (v/v)]	K_M (μM)	Relative V_{max}[a]
$pp60^{src}$	10	41.6	100
$pp60^{src}$	0	41.0	21.4

[a] V_{max} values are reported as a percentage of velocity observed with $pp60^{scr}$-derived peptide substrate at 100 to 0% (v/v) ethanol.

ion atmosphere and then to the binding of the substrate [55]. Final arguments for explaining the observed behavior have not been found yet.

In contrast, transaminases are very useful catalysts for amino acid synthesis, but are generally more complex and require special expertise compared to proteases and lipases [51].

The possibility to overcome product inhibition for a ω-transaminase has been investigated by applying one- and two-phase systems [56]. Several organic solvents were tested for the enzymatic resolution reaction of α-methylbenzylamine (α-MBA), but ethyl acetate and cyclohexanone as organic solvents yielded best results for enzyme activity and biocompatibility.

The usage of a two-phase system (Figure 33.13) for transaminase-catalyzed reaction has the major advantage of an *in situ* substrate and product removal from the organic phase. Due to this the acetophenone concentration is kept at a very low level, preventing product inhibition and leading to high reaction rates.

Additionally, the easy recovery of the chiral amine can be accomplished by adjusting the pH of the aqueous phase.

The phase ratio, expressed as the volume fraction of organic phase, can also lead to much higher reaction rates (Figure 33.14). Compared with the standard system (aqueous system), the reactivity increases ninefold at a value of 0.2, which is a further advantage of the two-phase system.

In analogy to hydrolytic enzymes like subtilisin that are also used for enzymatic resolution of racemic amines, transaminases are of minor interest in nonaqueous reaction systems [51].

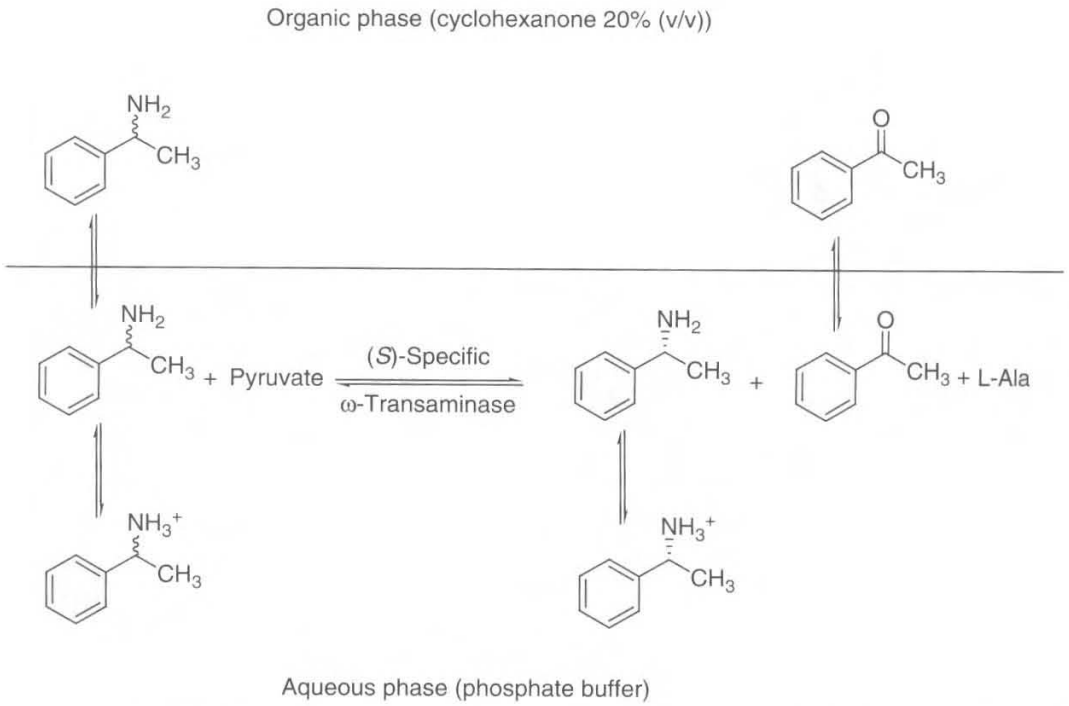


FIGURE 33.13 Two-phase-catalyzed enzymatic cleavage of α-methylbenzylamine using a ω-transaminase.

33.4 EC 3: HYDROLASES

The hydrolase family is classified in EC 3 and represents a group of enzymes that catalyzes bond cleavage by reaction with water. Several advantageous characteristics of these hydrolytic enzymes give them a high biotechnological potential and make them of special interest particularly with regard to their application in organic chemistry: (i) lack of sensitive cofactors; (ii) broad substrate specificity; (iii) high stereoselectivity; (iv) catalysis of several related reactions, such as condensation and alcoholysis; and (v) commercial availability [57].

Within the group of hydrolases, lipases (EC 3.1.1.3) stand amongst the most important biocatalysts, carrying out novel reactions in both aqueous and nonaqueous media. This is

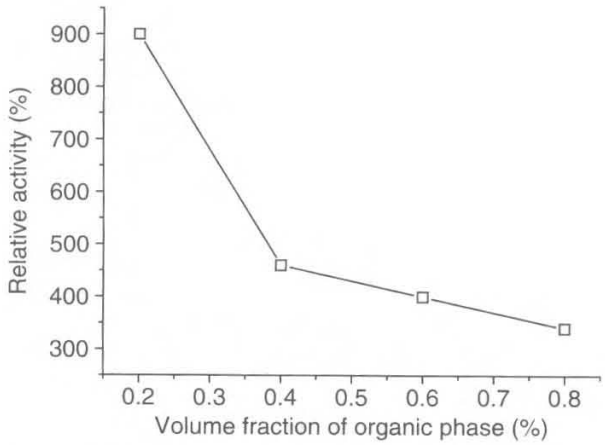


FIGURE 33.14 Enzyme activity in contrast to volume fraction of organic phase.

TABLE 33.5
Important Areas of Industrial Applications of Lipases

Industry	Examples of Use
Food industry	Bakery, diary: flavor improvement, transesterification of fats and oils, hydrolysis of milk fat [140,141]
Paper and wood industry	Bleaching of wood and recovered paper [142]
Pharmacy	Synthesis of chiral intermediates with high enantiomeric excess [143,144]
Medicine	Application in biosensors for identification of specific lipids (diagnostic of cardiovascular diseases) [145]
Detergent industry	Cleavage of fats in laundry [141,146]
Cosmetic industry	Surfactant synthesis and flavor synthesis [147]
Environment	Wastewater treatment, conditioning of waste fat and oils [148]
Agricultural economy	Pesticide synthesis [149]
Chemistry	Polyethylene terephthalate (PET) synthesis, bioconversions, seperation of enantiomers, oleochemistry [59,140,150–153]
Oleochemistry	Regioselective hydrolysis, transesterification, enantioselective processes; biodiesel, lubricants [141,154]

primarily due to their ability to utilize a wide spectrum of substrates, their high stability toward extremes of temperature, pH, and organic solvents, and their chemo-, regio-, and enantioselectivity. Lipases catalyze a wide range of reactions including hydrolysis, interesterification, alcoholysis, acidolysis, esterification, and aminolysis. They are used in a variety of biotechnological fields such as food and dairy, detergent, and pharmaceutical, agrochemical, and oleochemical industries (Table 33.5). Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts. Due to the tremendous potential of lipases for exploitation in biotechnology this section will mainly review this group of hydrolases.

Lipases are ubiquitous in nature: they occur in plants, animals, and microorganisms, and are primarily responsible for the hydrolysis of triglycerides with concomitant production of free fatty acids and glycerol. Many lipases are excreted extracellularly by fungi and bacteria, which makes their large-scale production particularly easy. In addition to their specific and limited function in metabolism, lipases play an important role in biotechnology: about 40% of all biotransformations reported so far have been performed with lipases [51]. In general, lipases are characterized by their unique feature of acting at water–organic interfaces, which distinguishes them from esterases [58,59]. Research in this field suggests that a lipid-induced conformational change alters the orientation of a lid that covers the enzyme active site. This phenomenon is commonly known as interfacial activation [57]. Consequently, lipase-catalyzed reactions are preferably conducted in two-phase systems.

Bearing in mind that the natural substrates of lipases are esters of an alcohol—glycerol—with an achiral acid, it is understandable that lipases are particularly useful for the resolution or asymmetrization of esters bearing a chiral alcohol moiety. On the basis of a thorough survey of the literature on chiral resolutions with lipases from *Candida rugosa* (CRL) and *Pseudomonas cepacia* (PCL), Bornscheuer and Kazlauskas proposed rules for the enantiopreference of these two enzymes on the spatial requirements of the substituents on the reagent [57]. The basics of "Kazlauskas rules" are shown in Figure 33.15 and literature has shown this rule to be highly predictive for lipase action on secondary alcohols, but less accurate for lipase-catalyzed transformations of primary alcohols and acids.

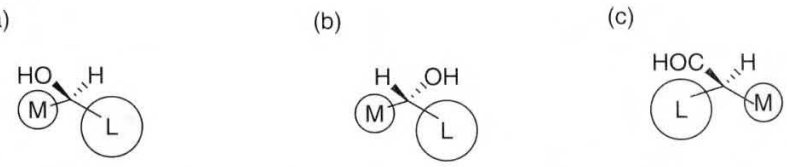


FIGURE 33.15 Scheme of the "Kazlauskas rule" to predict which enantiomer of (a) a secondary alcohol, (b) a primary alcohol, and (c) a carboxylic acid reacts faster in lipase-catalyzed reactions. M, medium-sized substituent, L, large-sized substituent.

Commercial lipase from *C. rugosa* is probably the most often used biocatalyst. This lipase has been applied for several selective hydrolysis reactions of esters of cyclic secondary alcohols, because its active site accepts larger substrates than those of other lipases [57]. One example to illustrate this point is the resolution of racemic 2,3-dihydroxy carboxylates and cyclohexane-1,2,3-triol esters by CRL [60,61]. PCL possesses a "smaller" active site than CRL and can be extremely selective on "slim" counterparts as was shown for the desymmetrization of some prochiral dithioacetal esters [62–66].

During the past decades the general opinion about the use of enzymes in organic reactions has changed. Conventional biocatalysis had mainly been performed in aqueous solutions, because enzymes were considered to be most active in water—their natural milieu. Subsequent years have seen the replacement of this prejudice by a more refined position initiated by the discovery that many enzymes are active in organic media.

Publications from the early 20th century and the 1930s already reported about biocatalysis in organic media, but most of this work was forgotten [67]. About 60 years ago researchers published first results about lipase-catalyzed esterifications in organic solvents containing approximately 8 to 12% of water [68,69]. However, it took more than 40 years to convince researchers that enzyme-catalyzed reactions are not only possible but also sometimes more convenient in organic solvents. Since the pioneering work of Cambou and Klibanov on this subject many publications have appeared on enzyme-catalyzed kinetic resolutions in organic solvents (especially lipase-catalyzed reactions) and on related topics like the use of organic cosolvents in water, reactions in solvent-free media or in compressed or supercritical gases, and the addition of salts or (thia)crown ethers to the lipase [70].

When performing lipase-catalyzed reactions in organic media, the ability to do an esterification reaction instead of hydrolysis is one advantage. It has been found that although lipases favor the same prochiral group in both cases, the two reactions yield opposite enantiomers. Figure 33.16 gives an example of the phenomenon: the acetylation of 2-benzylglycerol with PCL yields the (*S*)-monoacetate, while hydrolysis of the diacetate with porcine pancreatic lipase (PPL) yields the (*R*)-monoacetate.

Another advantage results from potential changes in the selectivity of lipases by applying different solvents, sometimes called medium or solvent engineering. Changes in the

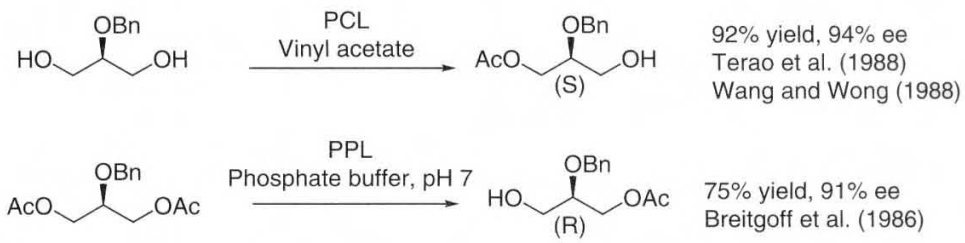


FIGURE 33.16 Acetylation of benzylglycerol with lipase of *Pseudomons cepacia* (PCL) and hydrolysis of the diacetate with porcine pancreatic lipase (PPL).

enantioselectivity after varying the solvent could be shown for PPL. Mori et al. found no enantioselectivity for the hydrolysis of seudenol acetate, but Johnston et al. reported moderate enantioselectivity ($E = 17$) in the acetylation of seudenol with trifluoroethyl acetate in ethyl ether [71,72]. These are only a few examples from the large volume of literature on lipase-catalyzed reactions in organic solvents. In order to give an overview of other techniques applied for these reactions the following sections will briefly discuss recent developments in other reaction systems.

33.4.1 Immobilization

Since the second half of the 20th century, investigations have focused on the development of immobilized enzymes in order to improve process economy by allowing the continuous or repetitive use and easy recovery of enzymes. Immobilization refers to the preparation of insoluble derivatives of enzymes, and has been performed by various methods including (i) noncovalent adsorption or deposition; (ii) covalent attachment; (iii) entrapment in a polymeric gel, membrane, or capsule; and (iv) cross-linking of the enzyme [73]. These techniques can dramatically affect enzyme properties such as pH dependence, temperature profile, and kinetics, and have often resulted in biocatalysts exhibiting significantly higher stability than the native enzyme.

Back in the 1950s, first immobilization methods for lipases have been reported. Although enzyme immobilization was dominated by physical methods during this time, first results of specific ionic adsorption like the binding of lipase on styrenepolyaminostyrene (Amberlite XE-97) have been published [74]. Unfortunately, those early-developed carriers were found to be not very suitable for enzyme immobilization. Since then many types of enzymes immobilized by different immobilization techniques have been found to exhibit higher stabilities, activities, and/or selectivities than native enzymes. Regarding lipases, entrapment in alkyl-substituted organic silane precursors has led to increased activity of these enzymes in organic solvents. The obtained sol–gels even show a significantly higher activity than freely dispersed enzymes [75]. The development of a novel technique for the immobilization of lipases by entrapping the enzymes within an aqueous solution in bead-shaped silicone elastomers ("static emulsion") led to an enhancement in enzyme activity by factor 31 for *C. antarctica* lipase A (CAL-A) and factor 250 for *Thermomyces lanuginosa* lipase in comparison to the native enzyme in hexane [76]. By entrapment of lipase–lipid complexes in *n*-vinyl-2-pyrrolidone gel matrix, Goto et al. succeeded in increasing the activity up to 50-fold in comparison to the native enzyme [77].

A variety of immobilization techniques like covalent bonding, entrapment, and adsorption can be applied to change the selectivity of lipases. For example, the *S*-selective lipase from *C. rugosa* presented a high enantioselectivity ($E = 400$) toward the *R*-isomer for the resolution of mandelic acid esters after covalent immobilization on glutaraldehyde supports [78].

In recent years, carrier-bound CLEAs have attracted increasing attention, due to their simplicity, broad applicability, high stability, and high volume activity. CLEAs of CRL with enhanced activity, stability, and defined particle size have been designed by impregnation of enzyme solution in a porous membrane of controlled pore size followed by subsequent aggregation and cross-linking [79].

33.4.2 Ionic Liquids

Over the last decade ILs have emerged as alternative reaction media for performing all types of reactions with sometimes remarkable results [80–82]. In 2000, Lau et al. investigated the reactivity of *C. antarctica* lipase B (CAL-B) in ILs, such as [BMIM][PF_6] and [BMIM][BF_4],

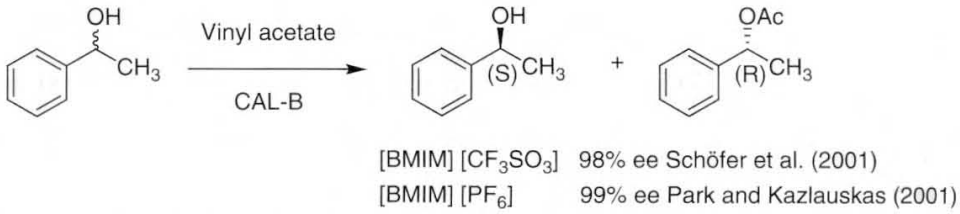


FIGURE 33.17 Lipase-catalyzed reaction in the presence of ionic liquids (ILs).

in comparison to conventional organic solvents [83]. They found similar reaction rates for all the reactions investigated. This work represents the second publication to demonstrate the potential use of ILs for enzyme catalysis and the first to show their use with lipases.

Since then several other lipases have been applied to catalyze reactions in systems with ILs as pure solvents, and as cosolvents with water or in two-phase systems. For example, lipase-mediated kinetic resolution of racemates in ILs provides extremely high enantiopurities of the products [84–86]. Kragl et al. investigated the kinetic resolution of 1-phenylethanol for a set of eight different lipases in ten different ILs with MTBE as reference [84]. To investigate transesterification vinylacetate was used as acetyl donor. The best results were obtained for CAL-B in [BMIM][CF_3SO_3], [BMIM][$(CF_3SO_2)_2N$], and [OMIM][PF_6]. Park and Kazlauskas reported good activities for CAL-B by using the ILs [BMIM][BF_4] and [BMIM][PF_6] in the same system (see Figure 33.17) [86].

Moreover, CAL-B has been found to exhibit impressive regioselectivities for the C-6 monoacetylation of β-D-glucose in [MOEMIM][BF_4]. Due to their solvation properties, ILs dissolve not only hydrophobic compounds but also hydrophilic compounds such as carbohydrates. Park and Kazlauskas reported the regioselective acylation of glucose with 99% yield and 93% selectivity in [MOEMIM][BF_4] (Figure 33.18). These values are much higher than those observed in the organic solvents commonly used for this purpose (reaction carried out in acetone: 72% yield, 76% monoacetylation) [86].

33.4.3 Reverse Micelles

The low water content necessary to favor synthesis reactions in organic media by lipases can be achieved by microencapsulation of the biocatalyst within reverse micelles. Reverse micelles consist of tiny aqueous droplets stabilized by surfactants in a bulk water-immiscible organic solvent. The biocatalyst remains soluble and active in the water phase, while reacting with water-insoluble or poorly soluble compounds present in the organic phase. The reversed micellar system has been proven to be highly suitable for lipase-catalyzed reactions. As the amount of aqueous phase is very small, lipases can catalyze transesterification and ester synthesis. Moreover, the system provides a high interfacial area and thus eliminates mass

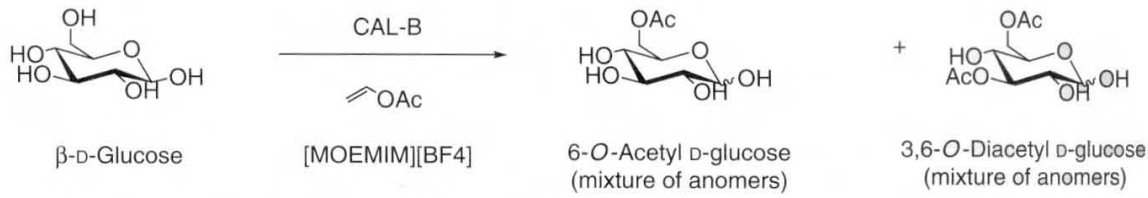


FIGURE 33.18 The acylation of glucose using *Candida antarctica* lipase B (CAL-B) in the ionic liquid (IL) [MOEMIM][BF_4]. (From Park, S. and Kazlauskas, R.J., *J. Org. Chem.*, 66, 8395–8401, 2001.)

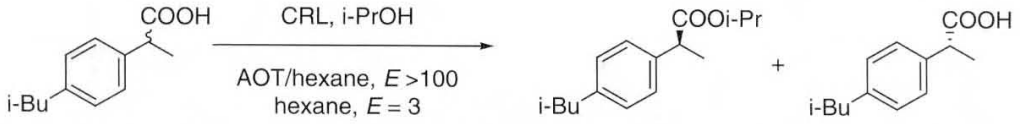


FIGURE 33.19 Esterification of ibuprofen by *Candida rugosa* lipase (CRL). (From Hedstrom, G., Backlund, M., and Slotte, J.P., *Biotechnol. Bioeng.*, 42, 618–624, 1993.)

transfer limitations. Many surfactants and solvents can be applied, but anionic surfactants, in particular AOT, have been proven to be best in lipase-catalyzed reactions [87].

The application of lipases in reverse micelles results in small changes in enzyme selectivity. In 1987 Bello et al. investigated the selectivity of CRL in the transesterification of triglycerides in reversed micelles and reported that CRL, which normally shows little fatty acid chain length selectivity, favored longer chain lengths in this system [88]. Moreover, the enantioselectivity of CRL can be increased by applying the biocatalyst in reverse micelles. Hedström et al. reported enantioselectivities of $E > 100$ for the CRL-catalyzed esterification of ibuprofen in reversed micellar systems in comparison with enantioselectivites of $E = 3$ in hexane (Figure 33.19) [89].

Many other applications of lipases in reverse micellar systems can be found in literature. Table 33.6 shows selected examples of reactions catalyzed by lipases with potential applications in food, pharmaceutical, and chemical industries as well as in the environmental area.

However, the recovery of products from surfactant-containing organic solvents still represents a problem that must be overcome before the reverse micellar system can be effectively applied at industrial scale. One possible solution might be the continuous operation in membrane reactors: an ultrafiltration membrane can be used to retain the micelles while the small molecules of substrate and products pass freely [90].

33.4.4 Supercritical Fluids

Lipase-catalyzed reactions have proven to be feasible in supercritical fluids (scF). These fluids represent substances heated above their critical temperature and compressed above their critical pressure. They exhibit properties similar to those of hydrophobic solvents, show

TABLE 33.6
Selected Examples of Reactions Catalyzed by Lipases in Reversed Micellar Systems

Enzyme/Source	System (Surfactant/Organic Solvent)	Reaction
Thermomyces lanugionsa	AOT Isooctane	Synthesis of ethyl-laurate [155]
Bacillus megaterium	AOT *n*-Heptane	Hydrolysis of *p*NPP [156]
Rhizopus delemar	AOT Isooctane	Hydrolysis of triolein [157]
Mucor javanicus	AOT Isooctane	Acylation of doxorubicin [158]
Candida lypolytica	AOT Isooctane	Esterification of octanoic acid with 1-octanol [159]

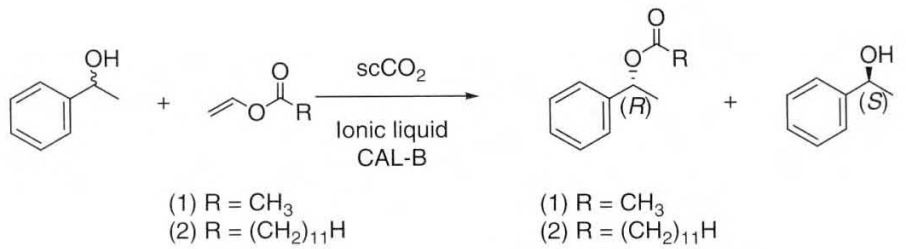


FIGURE 33.20 Lipase-catalyzed kinetic resolution in $scCO_2$. (From Reetz, M.T. et al., *Adv. Synth. Catal.*, 345, 1221–1228, 2003.)

rapid mass transfer due to low viscosity, and allow a simple downstream processing by evaporation. Moreover, their solvation properties can be changed by changing the pressure.

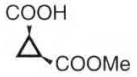
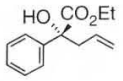
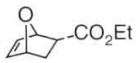
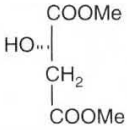
The use of supercritical carbon dioxide is probably the most common way to conduct enzyme-catalyzed reactions in scFs, because it is nonflammable, nontoxic, cheap, and reaches the supercritical state at 31.1°C. The first application of lipase-catalyzed reactions in scFs dates back to 1986. Nakamura et al. reported about the *Rhizopus oryzae* lipase (ROL)-catalyzed interesterification of triolein and stearic acid to 8% conversion in $scCO_2$ [91]. Since then researchers have examined a wide range of reactions and observed changes in conversion, enantioselectivity, and lipase stability similar to those in organic solvents. For example, Reetz et al. found high enantioselectivities for the lipase-catalyzed kinetic resolution of chiral racemic secondary alcohols (Figure 33.20) [92]. Lozano et al. and Reetz et al. reported the immobilization of CAL-B in ILs (Section 34.4.5), whereas substrates and products are dissolved in a second phase formed by supercritical CO_2 [93,94]. Further examples for lipase-catalyzed reactions in scFs can be seen in Table 33.7.

To summarize, this chapter aims to deliver a short insight into the immense possibilities of lipase application in organic synthesis. Future research will focus on the development of a new generation of lipases by extensive screening and genetic manipulations in order to create task-specific lipases for special applications. Protein-engineering methods will help to make

TABLE 33.7
Reactions in Supercritical Fluids (scFs) Catalyzed by Lipases

Enzyme/Source	System (Surfactant/Organic Solvent)	Reaction
Free and immobilized lipases: *Rhyzomucor meihei, Pseudomonas fluorescens, Rhizopus javanicus, R. niveus, Candida rugosa*	$scCO_2$ and scPropane	Ester synthesis: oleyl oleate [160]
Free and immobilized lipases: *C. antarctica, Mucor miehei*	$scCO_2$ and ionic liquids	Synthesis of glycidyl esters: kinetic resolution of *rac*-glycidol [161]
C. antarctica lipase (Novozyme 435)	scMethanol, scEthanol, and $scCO_2$	Synthesis of biodiesel [162]
Immobilized lipase: *M. miehei*	$scCO_2$	Hydrolysis of blackcurrant [163]
Immobilized lipase: *C. antarctica*	$scCO_2$	Butyl butyrate synthesis [164]

TABLE 33.8
Selected Pig Liver Esterase (PLE)-Catalyzed Reactions

Reaction	Substrate	Enzyme Source
Asymmetrization of nonchiral substrates [98]		PLE
Optical resolution of racemates [165]		PLE
Separation of endo/exoisomers [166]		PLE
Regioselective hydrolysis of ester groups [167]		PLE

lipases versatile industrial biocatalysts by enabling the production of large amounts of recombinant enzymes and the improvement of their biochemical and catalytic features.

33.4.5 Other Hydrolytic Enzymes

Esterases (carboxylester hydrolases, EC 3.1.1.1) catalyze, like lipases, the hydrolysis of carboxylic acid esters but, in contrast to lipases, only a few esterases have practical use in organic synthesis. Most of the esterase-catalyzed reactions in literature have been performed by the esterase isolated from pig liver (PLE) [95]. In contrast to lipases, PLE shows highest activity in aqueous buffered or two-phase systems, and usually does not accept highly hydrophobic substrates. Thus, selectivity tuning is more or less limited to the addition of up to 20% of polar protic water-miscible cosolvents like methanol, *tert*-butanol, DMSO, acetone, or acetonitrile [96]. In 1997 Ruppert and Gais succeeded to enhance the activity of PLE in organic solvents after colyophilization of the esterase with methoxypolyethylene glycol [97].

Esterases are in general very useful biocatalysts for the production of chiral intermediates through hydrolysis reactions. Examples include the asymmetrization of prochiral substrates and the optical resolution of racemates [98,99]. Esterases have also been used to separate endo/exo-mixture and for the regioselective hydrolysis of an ester group in the presence of a second ester function (Table 33.8).

Proteases are the last group of hydrolases to be mentioned in this review because they represent one of the three largest groups of industrial enzymes and find application in detergents, leather, food, and pharmaceutical industries, as well as bioremediation processes [100,101].

The most important commercial proteases are subtilisin, α-chymotrypsin, and—to a lesser extent—trypsin, pepsin, papain, and penicillin acylase. In the field of organic synthesis, two main applications of proteases can be found: (i) the enantioselective hydrolysis of carboxylic acid esters, where they seem to retain a preference for the hydrolysis of that enantiomer, which mimics the configuration of an L-amino acid more closely; and (ii) the synthesis of di- and oligopeptides by coupling of *N*-protected amino acids and peptide esters. The latter has been carried out on an industrial scale by Tosoh Corporation (Japan) for the thermolysin catalyzed synthesis of aspartame (Figure 33.21) [102,103].

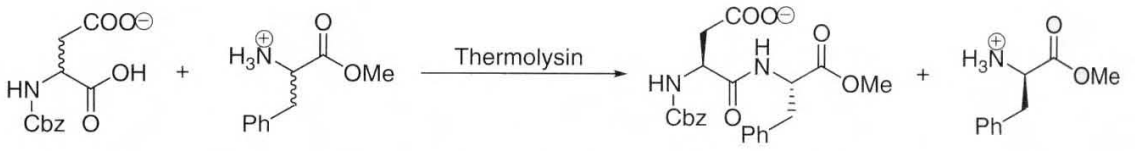


FIGURE 33.21 Commercial process for the production of aspartame by Tosoh Corporation (Japan).

TABLE 33.9
Subclasses of the Lyases

	EC 4 Lyases
4.1	Carbon–Carbon lyases
4.2	Carbon–oxygen lyases
4.3	Carbon–nitrogen lyases
4.4	Carbon–sulfur lyases
4.5	Carbon–halide lyases
4.6	Phosphorous–oxygen lyases
4.99	Other lyases

33.5 EC 4: LYASES

Apart from oxidoreductases and hydrolases, lyases are the most frequently used enzyme class [51]. They are divided into several subgroups, which are shown in Table 33.9 [50].

These enzymes catalyze several bond formation and cleavage reactions and have found extensive usage in several large-scale applications [104]. Two important examples, shown in Figure 33.22—(a) acrylamide synthesis and (b) *N*-acetylneuraminic acid—illustrate their use in the synthesis of bulk and fine chemicals.

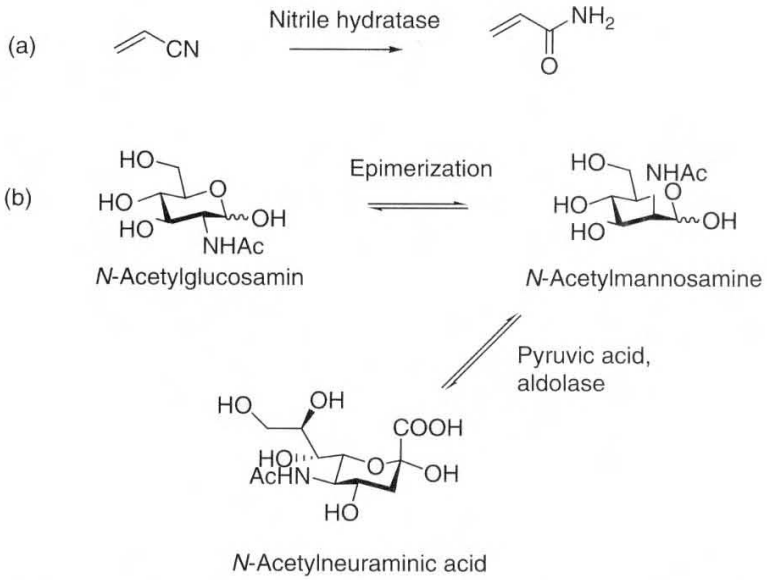


FIGURE 33.22 Large-scale applications of lyases. (From Wandrey, C., Liese, A., and Kihumbu, D., *Org. Proc. Res. Dev.*, 4, 286–290, 2000.)

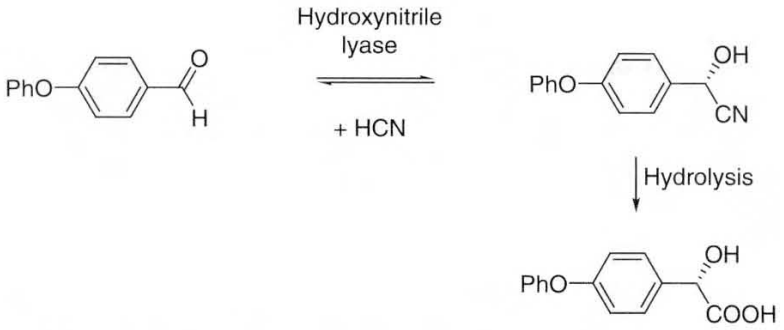


FIGURE 33.23 α-Hydroxy-carboxylic acids synthesis using hydroxynitrile lyases.

The cyanohydrin formation using hydroxynitrile lyases (HNLs) from several sources has produced enormous interest in the last decade. Important follow-up products are α-hydroxy-carboxylic acids, which are easily accessible using these enzymes (Figure 33.23), and have also been applied in large-scale operations [105].

33.5.1 Nitrile Hydratases

Nitrile hydratases hydrolyze nitriles selectively to the resulting amides. An important example is the enzymatic formation of acrylamide, see also Figure 33.24. The amide can be converted afterwards to the corresponding carboxylic acid. This two-step approach can also be simplified by using a nitrilase (Figure 33.24), yielding in one step the desired carboxylic acid [51].

In amide synthesis, using a nitrile hydratase, an important factor is the stability of the biocatalyst related to the rising product concentration [>50% (w/v) acrylamide], causing a rapid enzyme deactivation [106].

Several nitrile hydratases have been adapted to these challenges in acrylamide synthesis, e.g., the nitrile hydratase from *Rhodococcus* sp. N-774 in 1987 [107,108]. In 1988 a much more stable enzyme has been purified from *P. chloroaphis* B23 that exhibited a higher stability and reactivity in synthesis. An even more resistant nitrile hydratase from *Rhodococcus rhodochrous* J1 has been found in 1993. This enzyme exhibited an enormous stability against a variety of organic solvents (Table 33.10) [106].

As shown in Table 33.10, the variant from *R. rhodochrous* exhibits the highest stability against the organic solvents and also an activating effect for ethylene glycol. It has been assumed that the outstanding stability derives from the very high molecular mass of 505 kDa (20 subunits), by suppressing the flexibility of the protein.

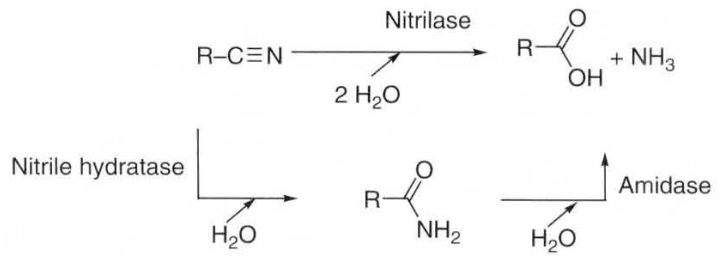


FIGURE 33.24 General pathway of the enzymatic hydrolysis of nitriles.

TABLE 33.10
Nitrile Hydratase Stability against Various Organic Solvents (Abstract)

Organic Solvent (50% (v/v))	Relative Activity (%)		
	Brevibacterium R312	*Pseudomonas chlororaphis* B23	*Rhodococcus rhodochrous* J1
None	100	100	100
Methanol	6	8	89
Ethanol	8	10	94
Acetone	10	12	66
Dimethyl sulfoxide	22	38	58
Ethylene glycol	64	55	136

In the past few years an enormous number of new nitrile-converting enzymes have been discovered, possessing high enantioselectivities and broad substrate ranges [109]. Nitrilases have also been sucessfully applied to organic solvents, like *Pseudomonas* sp. DSM 11387, as shown in Table 33.11 [110].

It can be seen that the relative activity is rising with increasing log *P* value, an observation that is comparable with other enzymes [111].

In the future, new immobilization techniques will provide even more stable enzymes. First attempts using the technique of CLEAs have been successfully arranged for nitrilases [37]. Additionally, the search for new enzyme sources and the modification of established nitrile-converting enzymes could lead to higher reactivities and selectivities. Potentially the combination of HNLs for obtaining cyanohydrins and the usage of nitrile hydratases for hydrolysis of the cyanohydrin in one step may also become an "interesting reaction" in the future [112,113].

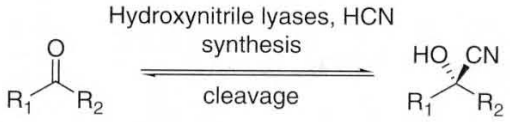
33.5.2 Hydroxynitrile Lyases

The HNLs reversely catalyze the enantioselective formation and cleavage of cyanohydrins. Several HNLs are known that convert (in the synthesis reaction) a broad range of aldehydes and ketones into the corresponding (*R*)- or (*S*)-cyanohydrins (Figure 33.25 gives examples of enzyme sources) [114].

Since the early works of Rosenthaler, in which he uses the enzyme preparation *emulsin* (from almond), several other enzyme sources have been intensely studied and reviewed for the cyanohydrin syntheses [114,115]. During the 1960s and 1970s HNLs were rediscovered and used in several large-scale applications. In the last decade an intense study on these enzymes led to the enclosing overlook about mechanism and structure for nearly all known HNLs.

TABLE 33.11
Stability of a Nitrilase in Organic/Aqueous Mixtures

Organic Solvent (50% (v/v))	log *P*	Relative Activity
No addition	—	100
1-Octanol	2.9	47
Octane	4.5	66
Hexadecane	8.8	97



R-selective	*Prunus amygdalus* (almonds), *Prunus serotina* (black cherry), *Linum usitatissimum* (linseed), *Prunus domestica* (plum), *Phlebodium aureum* (goldfoot fern)
S-selective	*Manihot ecsculenta* (manioc), *Hevea brasiliensis* (rubber tree), *Sorghum bicolor* (millet), *Annonia muricata* (guanabana)

FIGURE 33.25 Hydroxynitrile lyase–catalyzed reactions.

They can be divided into FAD-dependent (e.g., *Prunus amygdalus*) and non-FAD-dependent (e.g., *Manihot esculenta*) HNLs [116].

Beginning with an emulsion of benzaldehyde in water, used by Rosenthaler, several different reaction systems have been developed. As an important example, the immobilized enzyme on various supports has to be mentioned and has found broad usability for various HNLs like the successful application to the HNL from *M. esculenta* (Table 33.12) [117–119]. The enzyme is adsorbed on nitrocellulose suspended in an organic solvent (e.g., diisopropylether). Using this approach several aldehydes and ketones can be converted with high yields and very good ee [120].

Even though a macroscopic suspension (enzyme support suspended in an organic solvent) is formed, this system behaves like a one-phase system (see Section 33.1). Due to this restriction, limitations like substrate and product inhibition may occur. This disadvantage can be solved by using the very effective two-phase approach, using a buffer phase containing the enzyme and a water-immiscible organic solvent containing substrates. Applying this system the substrate and product concentration in the aqueous phase is very low, resulting from the partition coefficients between organic and aqueous phases.

Using this approach, several aldehyde and ketone cyanohydrins were easily accessible using the HNL from *Hevea brasiliensis* (Table 33.13) in a two-phase system consisting of a buffer and MTBE [121]. Recently, Lou et al. presented the first hydroxynitrile lyase–catalyzed reaction with encouraging results using the HNLs from *P. amygdalus* (*Pa*HNL) and *H. brasiliensis* (*Hb*HNL) in the IL–buffer two-phase system [122].

TABLE 33.12
Cyanohydrin Synthesis Using Adsorbed Wild-Type Hydroxynitrile Lyase from *Manihot esculenta*, Abstract

Substrate	Reaction Time (h)	Conversion (%)	ee (*S*) (%)
Benzaldehyde	0.5	97	99
2-Chlorobenzaldehyde	1	96	98
3-Phenoxybenzaldehyde	6.25	47	96
Decanal	17	65	78
Acetophenone	3	13	78
2-Thiophen	0.5	75	97

TABLE 33.13
Two-Phase System in Hydroxynitrile Lyase–Catalyzed Synthesis

Substrate	Water-Immiscible Solvent	Conversion (%)	ee (S) (%)
Benzaldehyde	MTBE	97	99
3-Phenoxy-benzaldehyde	MTBE	99	99
Propenal	MTBE	92	98
Benzaldehyde (*Pa*HNL)	PMIM BF_4	99	97 (*R*)
Benzaldehyde (*Hb*HNL)	PMIM BF_4	99	99

Furthermore, with the HNL from *H. brasiliensis* it has been demonstrated that the enzymatic reaction is performed only at the interfacial area and not in the aqueous bulk phase. This has been observed for the mandelonitrile cleavage by a blockade of the interface between diisopopylether and buffer [123].

Due to several new discovered HNLs and large-scale applications for cyanohydrins, using established enzymes, the observation of this enzyme class will proceed. Additionally, only a few reports were published using ILs in lyase-catalyzed reactions, especially for HNLs [122]. The research interest in the field of "enzymes in nonconventional media" will probably rise in the next few years. Also the usage of new molecular-biological attempts will gain more interest, with techniques like directed evolution. For example, a single residual replacement improves the folding and stability of a HNL from *M. esculenta* [125].

33.5.3 Aldolases

The usability of directed evolution in aldolase synthesis has been shown for a fructose bisphosphate aldolase. After four rounds of directed evolution using DNA shuffling of the *fda* genes from *E. coli* and *Edwardsiella ictaluri* the resistance against various organic solvents and also the thermostability have been increased [126].

The wild-type enzyme (*E. coli*) lost 40 to 90% of activity, whereas the variant activity remained nearly constant for an additional 20% (v/v) organic solvent (Figure 33.26). For a

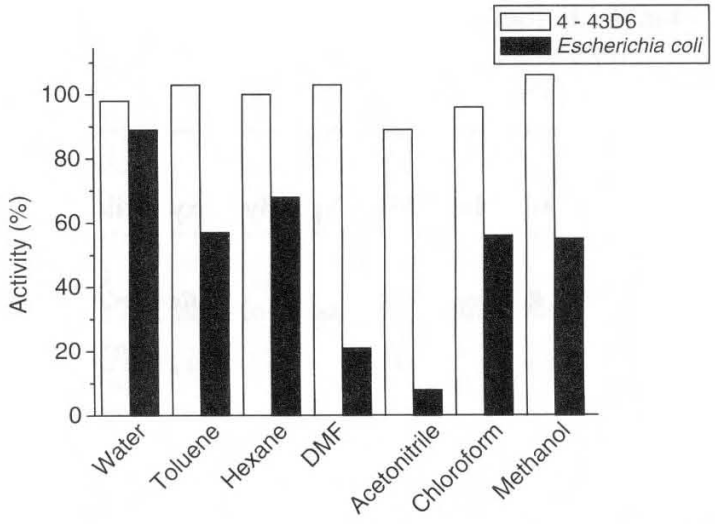


FIGURE 33.26 Irreversible inactivation, caused by the organic solvent [20% (v/v)].

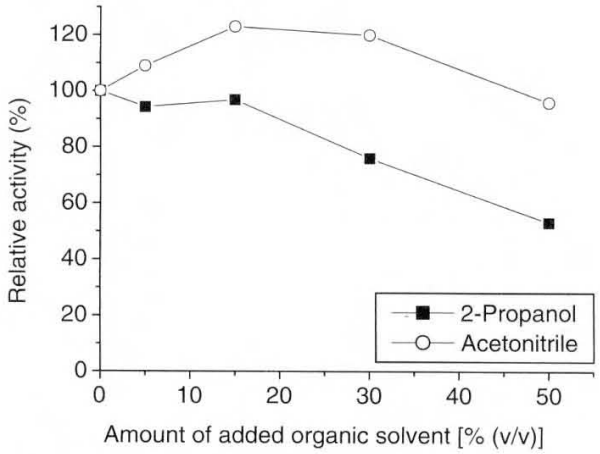


FIGURE 33.27 Influence of 2-propanol and acetonitrile on the aldolase from *Hyperthermophic archaea*.

heat-stable aldolase from *Methanococcus jannaschii* also a slight activating effect has been observed. By an addition of 15% (v/v) acetonitrile the relative activity increased to 23%, as shown in Figure 33.27 [127].

This stability against organic solvents may also be valuable for less-soluble substrates in enzyme catalysis in organic synthesis. A rabbit muscle aldolase (RAMA) showed an impressive stability with several water-miscible organic solvents (Figure 33.28).

Only for a few water-miscible organic solvents the enzyme activity was significantly reduced, whereas the water-immiscible organic solvents showed only a low decrease (maximum 50%).

As many substrates for aldolases are well soluble in water, the use of nonconventional media will be of limited interest.

33.6 EC 5: ISOMERASES

Isomerases catalyze isomerization reactions like racemization, epimerization, and rearrangement of different substrates. The utility of such enzymes is very low in industry because, on the one hand, there are only a few commercially available and, on the other hand, the

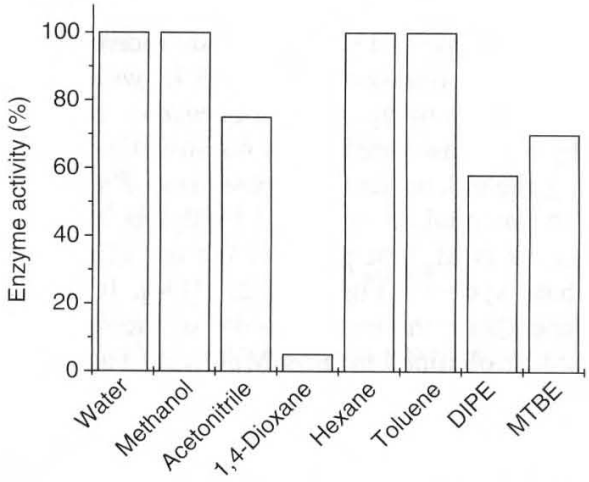


FIGURE 33.28 Rabbit muscle aldolase (RAMA) activity after 24 h of incubation at 20% (v/v), abstract.

TABLE 33.14
Subclasses of the Isomerases

	EC 5 Isomerases
EC 5.1	Racemases and epimerases
EC 5.2	*cis–trans*-Isomerases
EC 5.3	Intermolecular Oxidoreductases
EC 5.4	Intermolecular transferases
EC 5.5	Intermolecular lyases
EC 5.99	Other isomerases

exigency of isomerization in industry is marginal. Therefore, only about 2% of the publications in the period between 1987 and 2003 dealt with isomerase-catalyzed synthesis [51]. Isomerases are further divided into six subclasses (Table 33.14) [50].

In the last 25 years of the 20th century most reports were dealing with racemases and epimerases, which can be used for the dynamic kinetic resolution to get 100% conversion [51]. Yagasaki and Ozaki published the production of γ-aminobutyrat [128]. D-Glutamic acid was produced from L-glutamic acid. L-Glutamate was converted to the DL-form by the recombinant glutamate racemase of *L. brevis* ATCC8287. Then L-glutamate in the racemic mixture was selectively decarboxylated to the product by L-glutamate decarboxylase of *E. coli* ATCC11246. This was successfully realized in a one-pot reaction. Before this, there were a few articles dealing with cofactor independence of glutamate racemase from *Lactobacillus* sp. Some racemases like alanine racemase need a cofactor to catalyze the enzymatic racemization. In the case of the alanine racemase, pyridoxal phosphate (PLP) is the necessary cofactor. In addition to the relatively well-studied alanine racemase, arginine racemase from *Pseudomonas* sp. and two serine racemases from *Streptomyces* sp. require PLP for their catalytic activity [129].

The epimerization of threonine could be observed by the new amino acid racemase from *P. putida*. As mentioned earlier this epimerization has been done in buffered aqueous system as well, with attention on cofactor dependence—again the cofactor is pyridoxal 5′-phosphate—and on substrate specificity [130].

A typical enzyme to employ dynamic kinetic resolution is the mandelate racemase. In combination with a second biocatalyst, it is possible to transfer a racemate into a single stereoisomer in 100% theoretical yield [131]. Mandelate racemase catalyzes the racemization of different substrates in aqueous solution (HEPES pH 7.5 with 3.3 mM $MgCl_2 \cdot H_2O$) at room temperature, with further reaction by lipase from *Pseudomonas* sp. [132].

Syntheses catalyzed by isomerases are usually not investigated in nonconventional media. But the *O*-acylation of (±)-mandelic acid by lipase from *Pseudomonas* sp. was achieved in diisopropylether, while the mandalate racemase–catalyzed racemization took place in the aqueous phase [133]. Kaftzik et al. first reported the use of mandalate racemase in ILs as cosolvent and in two-phase systems (Figure 33.29) [134]. It is shown that the activity of the racemase strongly depends on the water activity of the reaction system. Activity of the mandalate racemase could be obtained in [BMIM][$OctSO_4$] at water activity a_w:0.75, and in biphasic systems consisting of water and [OMIM][PF_6] in a ratio of 1:10.

Isomerases will probably not find much consideration in organic synthesis taking place in nonconventional media in the future. Because of their scarce availability and limited purpose in biocatalytical synthesis, there are only a few applications.

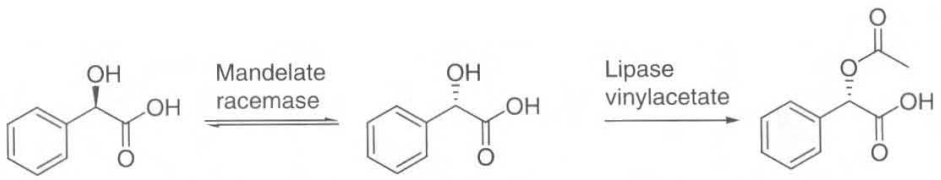


FIGURE 33.29 Deracemization of (±)-mandelic acid through a lipase-mandelate racemase two-enzyme system: *Pseudomonas* sp. lipase catalyzes *O*-acylation of (±)-mandelic acid and mandelate racemase–catalyzed racemization of remaining unreacted (*R*)-mandelic acid. (From Kaftzik, N. et al., *Mol. Catal. A Chem.*, 214, 107–112, 2004.)

33.7 EC 6: LIGASES

Ligases (synthetases) are classified in group 6 by the EC and represent a class of enzymes that catalyze the formation of bonds between two substrate molecules. They are further divided into subclasses according to the type of bond formed (Table 33.15).

The synthesis reaction catalyzed by ligases requires the hydrolysis of a nucleoside triphosphate such as adenosine triphosphate (ATP). In the field of ligases, examples of industrial biocatalysis are more or less missing [104]. This is further indicated by the fact that only about 1% of research from 1987 to 2003 has been performed with enzymes from the class of ligases [51]. One of the few examples for the biotechnological application of an enzyme from the group of ligases was described in 1998 by Aresta et al. [135]. They reported about a cheap, fast, and easy method for the phosphorylation of phenol at room temperature and subatmospheric pressure of CO_2: catalyzed by phenyl phosphate carboxylase, the synthesis of 4-OH benzoic acid from phenol and CO_2 with 100% selectivity was achieved (Figure 33.30).

Ligases also play an important role in the field of genetic engineering: DNA ligases (6.5.1.1) have become an indispensable tool for generating recombinant DNA sequences in modern molecular biology. By the development of a variety of nucleic acid–based detection systems for genetic disorders as well as for bacterial, viral, and other pathogens, ligases have been applied in a number of DNA amplification methods including polymerase chain reaction, self-sustained sequence replication, Q-beta replicase, and ligase chain reaction.

The discovery of DNA ligases and the biochemical studies of the ligase reaction by Lehman et al. were the first reports about host and bacteriophage-induced DNA ligases from eubacteria [136]. Subsequently, related enzymes from a wide range of organisms have been identified and studied. Today it is known that DNA ligases represent a large family of evolutionarily related proteins that play an important role in many essential reactions within the living cell including replication, recombination, and repair of DNA in all three kingdoms

TABLE 33.15
Subclasses of the Ligases

EC 6 Ligases	
EC 6.1	Forming carbon–oxygen bonds
EC 6.2	Forming carbon–sulfur bonds
EC 6.3	Forming carbon–nitrogen bonds
EC 6.4	Forming carbon–carbon bonds
EC 6.5	Forming phosphoric ester bonds
EC 6.6	Forming nitrogen–metal bonds

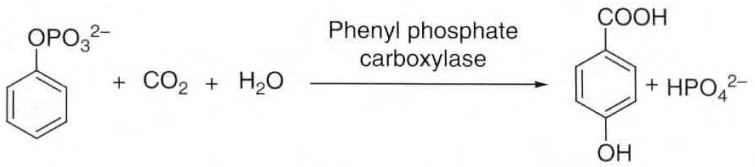


FIGURE 33.30 Synthesis of 4-OH benzoic acid from phenol and CO_2 by phenyl phosphate carboxylase. (From Aresta, M. et al., *Tetrahedron*, 54, 8841–8846, 1998.)

of life. They catalyze the formation of phosphodiester bonds at single-stranded breaks (nicks) between adjacent 3′-hydroxyl and 5′-phosphate termini in double-stranded DNA by using either ATP or NAD^+ as a cofactor [136,137].

As the presence of NAD^+-dependent DNA ligases is restricted to eubacteria, this fact makes them an attractive target for novel antibiotics. For example, bacterial NAD^+-synthetase (EC 6.3.5.1) catalyzes the last step in both the *de novo* biosynthetic and salvage pathways for NAD^+ and thus plays an essential role in the life cycle of bacteria. In order to find novel inhibitors for this enzyme for future antibacterial drug development, an enzymatic assay was developed by Yang et al. for the purpose of screening compounds for enzyme inhibition [138]. To improve the solubility of the compounds screened, the water-miscible organic solvent dimethyl sulfoxide (DMSO) was added to the assay buffer as cosolvent. Although no effects could be observed on behalf of the enzyme activity, concentrations of 2.5% (v/v) DMSO led to changes in the stability of the dimer and its unfolding mechanism.

Although bacterial NAD^+-dependent DNA ligases have been studied for more than 30 years, surprisingly few genetic and biochemical details are known about their regulation. In order to find new and interesting targets for future antibacterial drug development further investigations will be needed.

To summarize, ligases represent a class of enzymes found ubiquitously in nature. Due to their involvement in numerous essential reactions within the living cell and their cofactor dependency, the catalytic activity of ligases is more or less restricted to aqueous media, though some might act in the presence of small concentrations of cosolvents as well. While the subclass of DNA-ligases has already found numerous applications in molecular biology, the discovery of other ligases, e.g., acetyl-coenzyme A carboxylase (ACC) (EC 6.4.1.2), will be useful for the investigation of future potential targets for drug discovery. ACC plays a crucial role in fatty acid metabolism in most living organisms. It catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA and represents an attractive target for the therapeutic intervention in the control of obesity and the treatment of metabolic syndrome [139].

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Publikation 6

“Hydroxynitrile lyase catalysed synthesis of enantiopure (S)-acetophenone cyanohydrins”

von Langermann, J.; Mell, A.; Paetzold, E.; Kragl, U.
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Anteile: **von Langermann, J. (80%)**; Mell, A. (5%); Paetzold, E. (5%); Kragl, U. (10%)

Einleitung zu Publikation 6

Aufbauend auf die Publikation 2 wurden die Grundlagen der lösungsmittelfreien Zweiphasensysteme und die praktische Anwendung derselben in einem Buchbeitrag detaillierter beschrieben. Während die Grundlagen mit der Publikation 2 identisch ist, wurde in dieser Arbeit der praktischen Anwendung die größere Aufmerksamkeit gewidmet.

contribution

Hydroxynitrile lyase catalysed synthesis of enantiopure (*S*)-acetophenone cyanohydrins

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Introduction

Chiral cyanohydrins are versatile intermediates in the synthesis of α-hydroxy acids, β-amino alcohols, amino nitriles, α-hydroxy ketones and aziridines. For the synthesis of enantiopure cyanohydrins, the use of hydroxynitrile lyases is currently the most effective approach.[1-4] By applying an organic solvent free system also thermodynamically hindered substrates can be converted with moderate to excellent yields. With the usage of the highly selective hydroxynitrile lyase from *Manihot esculenta*, the synthesis of several acetophenone cyanohydrins with excellent enantioselectivities were developed (figure 1). (*S*)-Acetophenone cyanohydrin was synthesised in a preparative scale.[5]

< INSERT FIGURE 1 >

Materials and Equipment

- acetophenone (40 ml, 0.34 mol)
- hydroxynitrile lyase from *Manihot esculenta* (350 kU)
  (purchased from Julich Chiral Solutions, A Codexis Company, Jülich, Germany)
  Hydroxynitrile Lyases from other sources (e.g. *Hevea brasiliensis*, *Prunus amygdalus*, etc.) may be also used in a similar procedure.
- sodium cyanide (128 g, 2.6 mol)
- 5 mol/l sulfuric acid in water (320 ml)
- de-ionised water (320 ml, 17.8 mol)
- 50 mmol/l citrate buffer pH=4.0 (750 ml)
- diisopropylether (300 ml)
- sodium sulphate (water free)

- 1 distillation equipment (with dropping funnel) for the distillation of hydrogen cyanide
- 1 electrochemical HCN-detector for continuous monitoring
- 1 reaction flask 1 l
- stirrer
- centrifuge

Procedure

Synthesis of Hydrogen Cyanide (HCN)

The required amount of HCN was freshly distilled in a well ventilated fume hood.

128 grams of sodium cyanide were dissolved in 320 ml de-ionised water and 320 ml of 5 mol/l sulphuric acid were combined dropwise within the distillation equipment and the resulting solution was heated up to 75°C. Formed hydrogen cyanide was immediately trapped at 5°C and dried with water free sodium sulphate, whereas the receiving flask was also cooled to 5°C. Total yield of hydrogen cyanide: 68 ml.

An electrochemical HCN detector (Micro III G203, GfG-Gesellschaft für Gerätebau mbH, Dortmund, Germany) was placed in the fume hood for continuous monitoring.

Synthesis of (S)-Acetophenone Cyanohydrin

The synthesis should be performed within a well ventilated hood.

750 ml of 50 mmol/l citrate buffer pH=4 was placed in a 1 l-reaction flask and thermostated to 5 °C. Afterwards the freshly distilled hydrogen cyanide and 40 ml acetophenone were added to the buffer and the mixture was thermostated again to 5 °C. The reaction was started with the addition of 350 kU Hydroxynitril lyase from *Manihot esculenta*. The reaction

mixture was vigorously stirred and the reaction was followed by gas chromatography until the equilibrium conversion of 22% was reached (ca. 1.5 h).

Afterwards the reaction mixture was extracted twice with 150 ml diisopropylether. In case of problems with the phase separation the reaction mixture should be centrifuged. The combined solutions were dried with sodium sulphate and the organic solvent was removed under reduced pressure. The pure (*S*)-acetophenone cyanohydrin was obtained by distillation under reduced pressure without racemisation or decomposition of the product.[6]

Final yield of (*S*)-acetophenone cyanohydrin: 5g (isolated yield: 10 %). purity: > 95 %; enantiomeric excess: 98.5 % (measurement by gas chromatography)

Analytical Methods

The conversion of acetophenone (and the derivatives) to acetophenone cyanohydrin (derivatives) as well as their enantiomeric excess were determined by gas chromatographic analysis with a Chiraldex capillary gas chromatography column (G-PN – g-Cyclodextrin, Propionyl) from astec using a CP3800 (Varian) with a flame ionization detector (FID). Carrier gas was helium at 2 ml/min. Temperature gradient: 80 °C for 0.5 min, rise with 10 8°/min to 130 °C and hold 130 °C for 15 min . The injector and detector temperatures were set to 250 °C.

Enzyme Assay

One Unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the cleavage of 1 mmol mandelonitrile per minute under assay conditions.

The enzyme activity was determined by following the cleavage of rac-mandelonitrile into benzaldehyde and HCN at 25 °C. The formation of benzaldehyde was measured

spectrometrically at 280 nm. The non-enzymatic cleavage reaction was monitored under identical conditions and subtracted.

Assay conditions: 700 µl citrate-phosphate buffer pH 5.0, 100 µl enzyme solution (dilution if required) and 200 µl mandelonitrile stock solution (60 mmol/l in citrate-phosphate buffer pH 3.5) were mixed in a cuvette with 1 cm path length and the increase of absorbance at 280 nm was measured for 2 min.

derivatisation procedure for the gas chromatography:

A sample (100 µl) of the suspension or 1 µl of the crude product was added to 100 µl of diisopropyl ether for the extraction. 50 µl of the organic phase (diisopropylether) were added to a mixture of 500 µl dichloromethane, 50 µl trifluoroacetic anhydride and 50 µl pyridine for the acetylation procedure. The mixture was directly injected into the gas chromatograph.

All waste solutions were collected and disposed with hydrogen peroxide.

acetophenone derivative cyanohydrin formation

Additionally, several other acetophenone derivatives were converted on a smaller scale (table 1).

< INSERT TABLE 1 >

Conclusion

The usage of organic solvent free systems can be applied to a wide range of acetophenone derivatives, whereas electronegative substituents (e.g. fluorine) facilitate high grade of

conversions and enantiomeric excess'. On the other hand, electropositive substituents (e.g. methoxy-) were not converted to the corresponding cyanohydrins.

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Figure 1

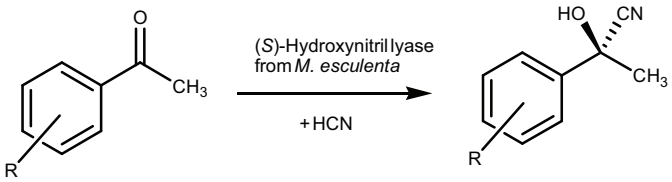

Table 1. acetophenone derivative cyanohydrin formation

acetophenone (AP) derivative	time / h	conversion / %	e.e.(*S*) / %
4'-F-AP	1.5	14	> 99
3'-F-AP	**3**	**48**	**> 99**
2'-F-AP	**3**	**71**	**> 99**
2',3',4',5',6'-F-AP	6	< 1	-
4'-Cl-AP	6	18	97
3'-Cl-AP	**6**	**23**	**97**
2'-Cl-AP	4.5	6	80
4'-Br-AP		solid	
3'-Br-AP	6	10	> 99
2'-Br-AP	6	9	68
4'-I-AP		solid	
2'-I-AP	6	< 1	-
4'-Me-AP	6	< 1	-
3'-Me-AP	6	< 1	-
2'-Me-AP	6	< 1	-
4'-MeO-AP		solid	
3'-MeO-AP	6	< 1	-
2'-MeO-AP	6	< 1	-
4'-NO_2-AP		solid	
3'-NO_2-AP		solid	
2'-NO_2-AP	**1.5**	**40**	**> 99**

4’-NH_2-AP		solid	
3’-NH_2-AP		solid	
2’-NH_2-AP	6	< 1	-
4’-OH-AP		solid	
3’-OH-AP		solid	
2’-OH-AP	6	< 1	-

reaction conditions: reaction time: 1.5 - 6h; 0.4 mmol acetophenone derivative: 2 mmol hydrogen cyanide; 1 ml citrate buffer pH=4.0 or pH=4.8, 5 °C, 450 U/ml, 400 rpm

solid – not determined due to the absence of an organic layer

9 Anhang

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Publikationsliste

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1. “Influence of water-miscible organic solvents on kinetics and enantioselectivitiy of the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis*”
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   Advanced Synthesis and Catalysis, 349, 1418-1424 (2007)
4. “A new (*R*)-selective Hydroxynitrile Lyase from Arabidopsis thaliana with an alpha/beta-Hydrolase fold”
   Andexer, J.; **von Langermann, J.**; Mell, A.; Bocola, M.; Kragl, U.; Eggert, T.; Pohl, M.,
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7. “Hydroxynitrile lyase catalysed synthesis of enantiopure (*S*)-acetophenone cyanohydrins”
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Präsentationen

1. *„The first R-selective Hydroxynitrile lyase with an α/β-Hydrolase fold"*; J. N. Andexer, J.-K- Guterl, **J. von Langermann**, A. Mell, T. Eggert, U. Kragl, M. Pohl; (Poster), New Directions in Molecular Genetics and Genomics, 9.-11.4.2008 (Poster)
2. *„Biokatalyse mit Hydroxynitril Lyasen - Unterdrückung der nicht-enzymatischen Cyanhydrinsynthese"*; **Jan von Langermann**, Udo Kragl; 41. Jahrestreffen Deutscher Katalytiker, Weimar, 27.-29.2.2008 (Vortrag)
3. *„Hydroxynitrile lyases in organic solvent free systems to overcome thermodynamic limitations"*; **Jan von Langermann**, Udo Kragl; 10. Frühjahrssymposium der Jungchemiker, Rostock, 27.-29.3.2008 (Poster)
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6. *"Influence of water-miscible organic solvents on a secondary alcohol dehydrogenase from Lactobacillus brecis"*; **Jan Schumacher**, Marrit Eckstein, Udo Kragl; 7th International Symposium on Catalysis Applied to Fine Chemicals, Bingen/Mainz, 23.-27.10.2005 (Poster)
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8. *"Arbeitsgruppe Technische Chemie"*; **Jan Schumacher**, Christian Vogl; ConNeCat-Partnering Event – Katalytische Verfahren zur Nutzung nachwachsender Rohstoffe, Frankfurt am Main, 25. 1. 2005 (Poster)

Selbständigkeitserklärung

Ich versichere hiermit, dass diese Dissertation selbstständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde. Die aus fremden Quellen direkt übernommenen Gedanken sind als solche kenntlich gemacht.

Die vorliegende kumulative Dissertation wurde bisher in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vorgelegt und auch nicht veröffentlicht.

Rostock, den 29.4.2008

Jan von Langermann und Erlencamp