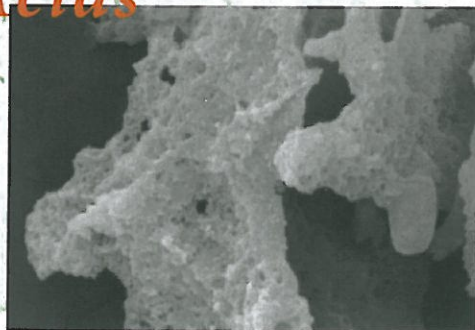


A Sustainable,
Two-Enzyme,
One-Pot Procedure for
the Synthesis of
Enantiomerically Pure

α -Hydroxy Acids



Andrzej Chmura

**A Sustainable, Two-Enzyme, One-Pot
Procedure for the Synthesis of
Enantiomerically Pure α -Hydroxy Acids**

Andrzej Chmura

Cover picture:

Manihot esculenta (cassava), source: <http://www.hear.org/starr/images/image/?q=090618-1234&o=plants>

SEM photograph of an *MeHnL* CLEA (author: Dr. Rob Schoevaart, CLEA Technologies, Delft, The Netherlands)

Cover design by Andrzej Chmura

A Sustainable, Two-Enzyme, One-Pot Procedure for the Synthesis of Enantiomerically Pure α -Hydroxy Acids

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof.ir. K.C.A.M. Luyben,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen
op dinsdag 7 december 2010 om 12.30 uur

door

Andrzej CHMURA

Magister inżynier in Chemical Technology,
Politechnika Wrocławska, Wrocław, Polen
en
Ingenieur in Chemistry,
Hogeschool Zeeland, Vlissingen, Nederland
geboren te Łańcut, Polen

Dit proefschrift is goedgekeurd door de promotor:

Prof. dr. R.A. Sheldon

Copromotor: Dr. ir. F. van Rantwijk

Samenstelling promotiecommissie:

Rector Magnificus	Voorzitter
Prof. dr. R.A. Sheldon	Technische Universiteit Delft, promotor
Dr. ir. F. van Rantwijk	Technische Universiteit Delft, copromotor
Prof. dr. I.W.C.E. Arends	Technische Universiteit Delft
Prof. dr. W.R. Hagen	Technische Universiteit Delft
em. Prof. dr. A.P.G. Kieboom	Universiteit Leiden
Prof. dr. A. Stolz	Universität Stuttgart
Prof. dr. V. Švedas	Lomonosov Moscow State University
Prof. dr. J.J. Heijnen	Technische Universiteit Delft, reserve lid

The research described in this thesis was financially supported by The Netherlands Research Council NWO under the CERC3 programme and by COST action D25.

ISBN/EAN: 978-90-9025856-0

Copyright © 2010 by Andrzej Chmura

All rights reserved. No part of the material protected by this copyright notice may be reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying, recording, broadcasting, or by any storage and retrieval system, without permission in writing from the author.

Contents

Chapter 1	Introduction	1
Chapter 2	Cross-Linked Enzyme Aggregates of the Hydroxynitrile Lyase from <i>Manihot esculenta</i> : Highly Active and Robust Biocatalyst	23
Chapter 3	Cross-Linked Enzyme Aggregates of Two Enzymes (combi-CLEA)	35
Chapter 4	Cross-Linked Enzyme Aggregates of Three Enzymes (triple-CLEA)	55
Chapter 5	A novel, One Cell, Two Enzyme Biocatalyst. The Biocatalytic Tests of Recombinant <i>E. coli</i> which Simultaneously Express an (S)-Oxynitrilase and a Nitrilase	77
Chapter 6	Nitrile Hydratase Activity of a Recombinant Nitrilase	87
Chapter 7	Characterization of a Nitrilase Derived from a Haloalkaliphilic Gammaproteobacterium <i>Halomonas nitrilicus</i> sp. nov.	103
Chapter 8	I Surfactant Catalyzed Hydrocyanations II Asymmetric, One-Pot, Chemo- Enzymatic Synthesis of Protected Cyanohydrins	129
Summary		151
Samenvatting		155
Dankwoord		159
List of publications		162
Oral presentations		163
Curriculum Vitae		165

1. Introduction

The discovery of optical isomerism dates back to 1815 when Jean-Baptiste Biot performed experiments using polarized light, passing it through various solutions. He noticed that when doing so, certain sugar solutions rotate polarized light. However, it took more than 30 years to understand the cause of this discovery. In 1848 Louis Pasteur performed the pioneering work on the separation of different structural forms of sodium ammonium tartrate, only later called optical isomers, discovering that their crystals are mirror images of each other. A few decades later, Van't Hoff proposed tetrahedral carbon and the notion that optical isomers are structural mirror images. This gave rise to a new discipline - stereochemistry. Since then, it started to evolve into the major field of intensive research that it is nowadays. In recent decades the synthesis of optically active compounds in enantiomerically pure form has gained a new impetus due to the recognition that enantiomers can interact differently with biological systems, on account of the chirality of the latter, and that in general only one of the enantiomers (the eutomer) exhibits the desired biological activity whereas the other enantiomer is at best ballast. This was already shown in the 19th century when Pasteur showed that a certain strain of a micro-organism could grow on one enantiomer of tartaric acid whereas the other was unconsumed. Often, however, the antipode of the eutomer (the distomer) inhibits the desired effect or may even cause severe side effects. Thalidomide (Figure 1), commercially known under different names, such as Softenon, is a dramatic example.

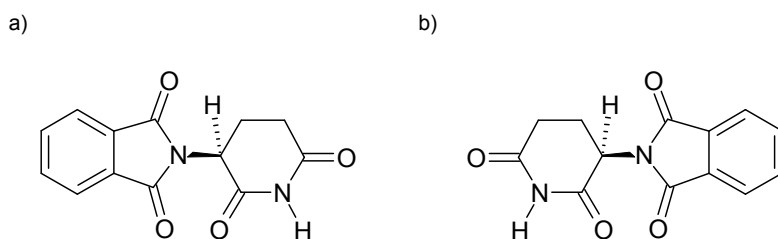


Figure 1. (*S*)-thalidomide (a) and (*R*)-thalidomide (b)

When thalidomide was introduced (antidepressant and sleeping aid) it turned out to be strongly teratogenic. This latter effect was subsequently attributed to the distomer, the (*S*)-enantiomer.^{1, 2} It is worth noting that in this particular case, administering (*R*)-

thalidomide would not solve the problem as thalidomide racemises fairly fast under physiological conditions.

Today, enantiomers are recognized as separate medical compounds. For example citalopram ((*R,S*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile) a known antidepressant drug used to treat mood disorders, is also available as the pure (*S*)-enantiomer, known as escitalopram. A head-to-head comparison of citalopram and escitalopram found the latter to be both more tolerable and more effective. Similar examples of eutomer vs. distomer behaviour of enantiomers are known in agrochemistry.

Enantiomerically pure α -hydroxy acids are an important class of compounds in both pharmaceutical and (agro) chemical applications (Figure 2). Due to their importance and their growing market, it is not surprising that tremendous efforts have been made to establish enantioselective routes for their production.³ However, in early applications, a set of natural α -hydroxy acids – known due to their origin as fruit acids; glycolic, malic, tartaric and citric acid and also mandelic acid (derived from sugar cane, apples, grapes, lemons and almonds respectively)⁴ or, lactic acid (the second smallest α -hydroxy acid, originally derived from sour milk⁵ or from fermentation of sugar with a *Lactobacillus*) were used.

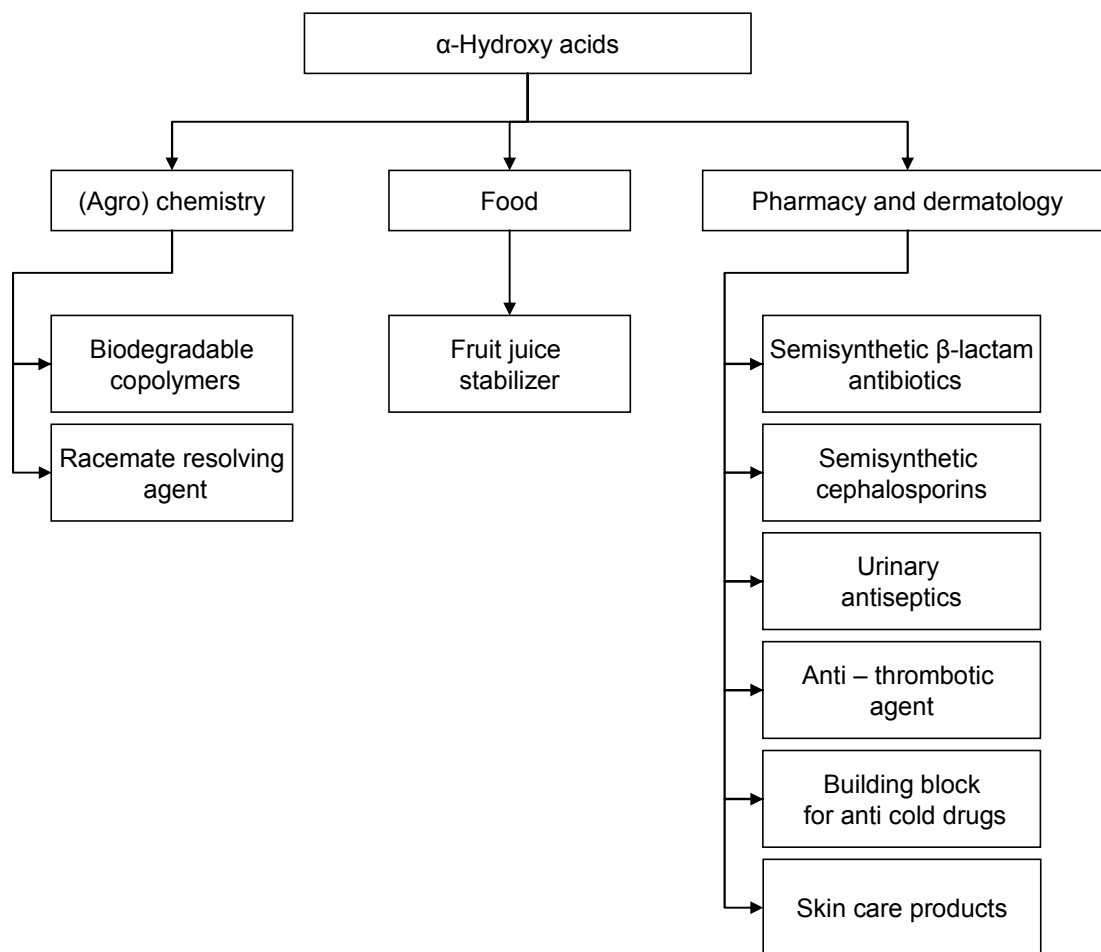


Figure 2. Applications of α -hydroxy acids.

Biodegradable copolymers containing (*S*)- or (*S,R*)-lactic acid are of great medical interest. These copolymers are physiologically acceptable and can be used as a composition having a biologically active material mixed therewith or entrapped within.⁶ More recently, polylactic acid has been introduced in general use. Mandelic acid has proven to be a convenient resolving agent for a large number of amines.⁷ There are also applications of α -hydroxy acids in foods and beverages where e.g. (*S*)-malic acid is used for the stabilization of fruit juices.⁷ By far the most versatile, important and recognizable applications of α -hydroxy acids can be found in the pharma industry. (*R*)-mandelic acid and (*R,S*)-mandelic acid are used as a versatile intermediate for pharmaceuticals such as e.g. semisynthetic β -lactam antibiotics^{3,8} or as urinary antiseptic⁹ respectively. Pfizer in turn, studies yet another α -hydroxy acid; (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid. This acid is one out of four building blocks of Rupintrivir. Rupintrivir shows rhinovirus protease inhibitor properties and it

can be used to treat the common cold.¹⁰ The structure of Rupintrivir and the synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid are discussed in more details in section 1.2.2 of this Chapter.

(*R*)-2-Chloromandelic acid is a building block for the industrial synthesis of the anti-thrombotic agent, (*S*)-clopidogrel (Plavix).^{11, 12} In 2006 global sales of Plavix reached $6.4 \cdot 10^9$ US dollars what made it the second largest drug with respect to sales.¹³ The medical importance and the sale volumes reflected on the efforts to industrialize the production of this medicine. Since the market launch of clopidogrel in 1993,¹⁴ several production methods were developed and Sanofi's contribution to the research on the synthetic production ways towards clopidogrel is undisputable. Clopidogrel was initially synthesized using (*R,S*)-2-chloromandelic acid as a starting material, giving after a three step reaction (*R,S*)-clopidogrel. The biologically active (*S*)- enantiomer of the latter racemate was obtained in a separate step via a resolution with camphor-10-sulfonic acid (Figure 3 a), b) respectively).

a)

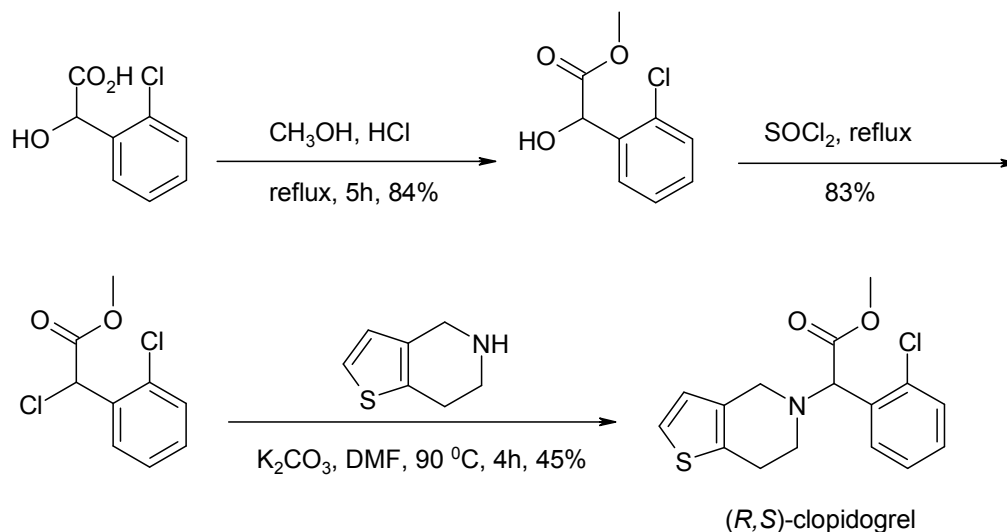


Figure 3a. Sanofi process; synthesis of (*R,S*)-clopidogrel.

b)

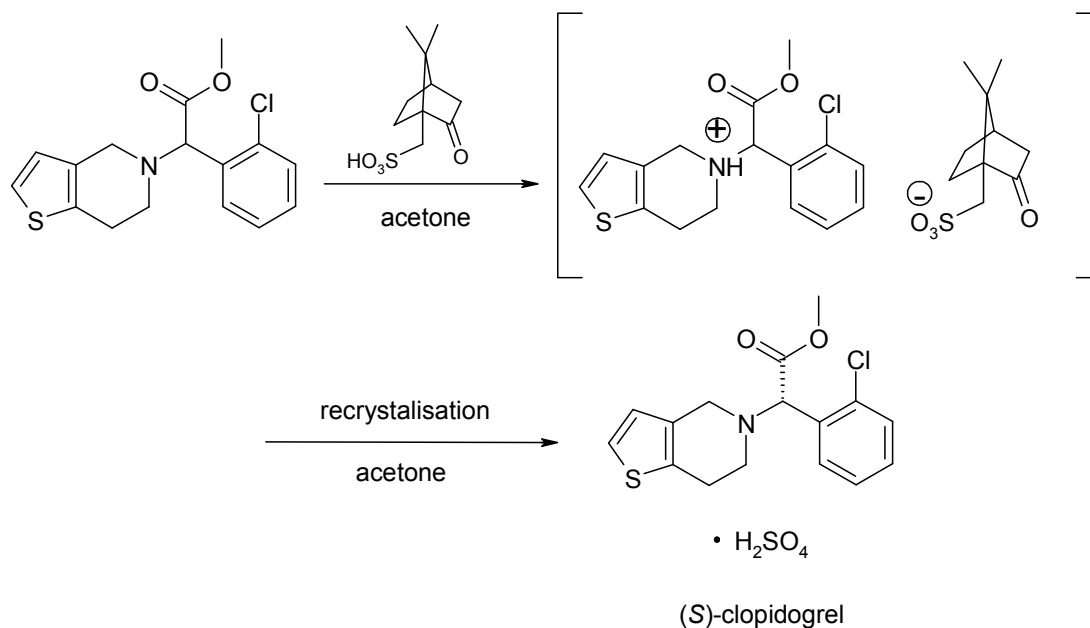


Figure 3b. Sanofi process; resolution of (*R,S*)-clopidogrel with camphor sulfonic acid (b).¹⁴

Over time, Sanofi improved the above processes, significantly simplifying it, and according to a recent paper,¹⁵ the drug is produced today from (*R*)-2-chloromandelic acid as a starting material giving enantiomerically pure (*S*)-clopidogrel (Figure 4), thus skipping the laborious resolution.^{14, 16, 17}

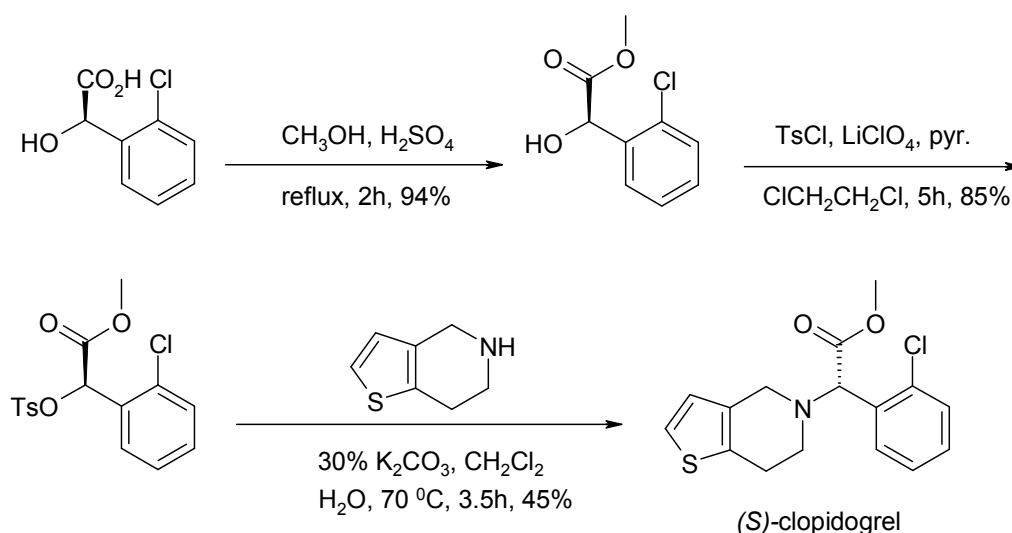


Figure 4. Sanofi process for enantiomerically pure (*S*)-clopidogrel with (*R*)-2-chloromandelic acid as the starting material.¹⁴

The above described processes are very similar, but differ in the leaving groups introduced and replaced in the second and third steps of the synthesis (chloride and tosylate respectively).

Yet, there exist other ways to produce (*S*)-clopidogrel. A chemical method starting with (*R,S*)-2-chlorophenylglycine¹⁴, an enzymatic process starting with (*R,S*)-2-chlorophenylglycine ester¹⁸ and a one-pot process starting with (2-chlorobenzyl)-(2-thiophen-2-yl-ethyl)amine hydrochloride developed by RPG Life Sciences¹⁴ are worth to be mentioned here.

In terms of applications, (*R*)-2-chloromandelic acid can be found to be one of the most interesting enantiomerically pure α -hydroxy acids. Hence, we wish to discuss in more details the chemo-enzymatic methods of its industrial synthesis. The production of (*R*)-2-chloromandelic acid is dominated by DSM Chemie Linz, Nippon Shokubai, and Clariant. All these companies use a similar two-step, chemo-enzymatic pathway (Figure 5).⁷

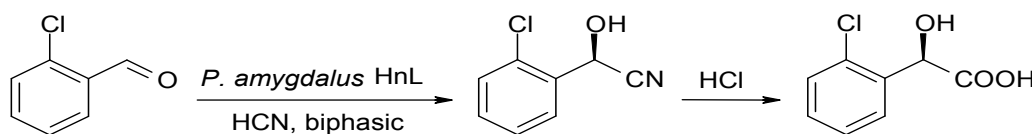


Figure 5. Oxynitrilase catalysed synthesis of (*R*)-2-chloromandelic acid. General production scheme used by DSM Chemie Linz, Nippon Shokubai and Clariant.

The first, enzymatic step employs the highly (*R*)-selective *P. amygdalus* oxynitrilase (HnL) and the efficiency of this step is crucial for the final product purity. During the reaction, cyanide anion is asymmetrically added to 2-chlorobenzaldehyde. While Nippon Shokubai and Clariant use native enzyme extracts, DSM Chemie Linz uses a mutated HnL (PaHnL5 mutant¹⁹ immobilized on Avicel,^{7, 19} resulting in 95% enantiomerically pure product at a space-time yield of 250 g·L⁻¹·d⁻¹²⁰). The following hydrolysis step, carried out after separation from the enzyme, is performed in all cases in 35% HCl at elevated temperatures. However, comparing to other manufacturers, the Nippon Shokubai biphasic hydrolysis process seems to be more efficient. The advantage of such an approach is a high purity of the final product that had to be recrystallized otherwise.

The potential of α -hydroxy acids in skin care products was recognized for the first time already centuries ago. The first, scientifically documented use of this group of compounds however, dates from the mid. '70s of the last century, when van Scott *et al.* reintroduced the use of α -hydroxy acids, recognizing their beneficial effects.^{21, 22} Today, most of the α -hydroxy acids used in cosmetics (over 200 different products in 1996 with the acid concentrations varying from 10-70% w/w^{23, 24}) are manufactured synthetically by industrial methods.^{21, 24, 25}

1.1. Chemical methods for the synthesis of enantiopure α -hydroxy acids

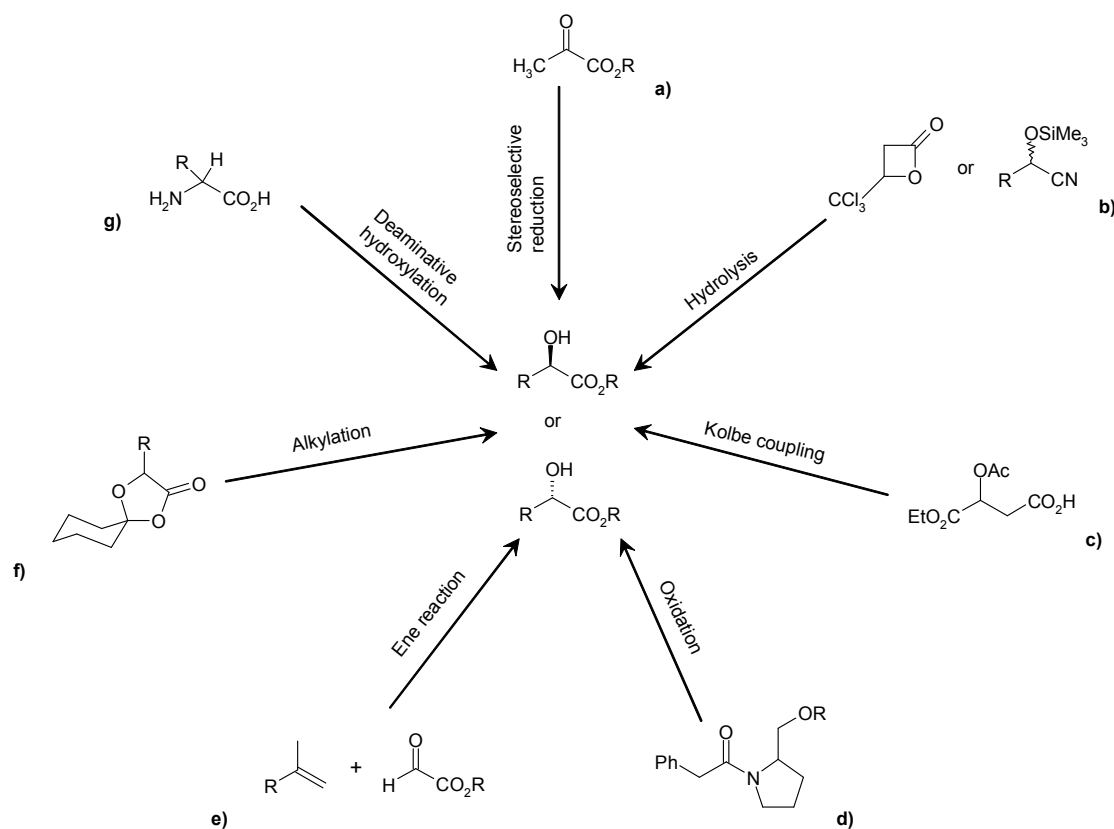


Figure 6. Selected chemical methods for obtaining enantiomerically pure α -hydroxy acids: a) stereoselective reduction of α -keto esters²⁷, b) hydrolysis of tri-chloro lactones^{28, 29} or chemical hydrolysis of silyl protected cyanohydrin⁷ (or alternatively enantiopure ketone cyanohydrins formed in enantioselective enzymatic hydrocyanation reaction,^{30, 31}), c) via Kolbe coupling^{32, 33}, d) oxidation of chiral esters³⁴, chiral amide³⁵, imide enolates³⁶ or 1,2-diols in alkaline solution³⁷, e) ene reactions of chiral glyoxylate esters³⁸, f) alkylation of chiral glycolate enolates³⁹⁻⁴¹, g) deaminative hydroxylation of α -amino acids⁴².

Over a hundred years of research on the synthesis of optically active compounds resulted in numerous ways to obtain such compounds. They can be divided into three groups: separation of a racemic mixture (resolution), isolation of chiral material from natural sources and stereoselective chemical and biochemical synthesis.^{2, 26} A number of chemical methods fall into the latter categories (Figure 6).

Other methods not pictured in the above figure are: acetoxylation of chiral carboxylic esters⁴³, ring opening of chiral glycidic esters², deamination of optically active α -amino acids^{26, 44}, via kinetic resolution of racemic α -hydroxy acids with glycolate oxidase (GOX) and simultaneous reduction with SnCl_2 ^{45, 46}. Chemical synthetic ways however, will not be further described here as they fall outside the scope of this thesis.

1.2. Enzymatic methods for the synthesis of enantiopure α -hydroxy acids

Apart from the chemical methods, the asymmetric formation of (aromatic) α -hydroxy acids can also be accomplished by choosing a biocatalytic process (fermentation⁴⁷ or enzymatic conversions) as key synthetic step. Enzymes are highly valuable catalysts and allow the manufacture of chiral chemicals on an industrial scale with high enantioselectivity, yield, volumetric productivity and little waste. The various synthetic, enzymatic pathways for synthesis of (*R*)-mandelic acid are described in a review by Gröger.³ Many enzymatic approaches towards enantiomerically pure (aromatic) α -hydroxy acids have been developed by several groups applying a broad range of concepts and can be categorized in four enzymatic routes (Figure 7). These concepts are based on different types of biotransformations covering hydrocyanation, chiral resolution processes as well as hydration and reduction reactions.^{3, 7}

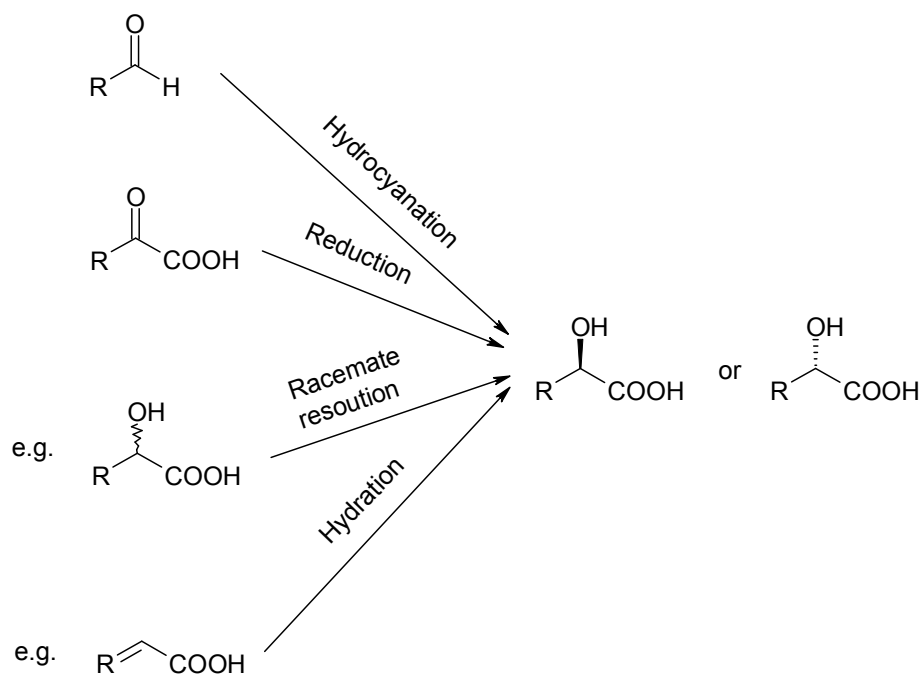
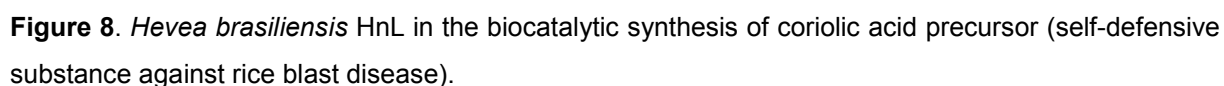


Figure 7. Enzymatic paths for synthesis of α-hydroxy acids.

1.2.1. Hydrocyanation

Enzymatic hydrocyanation processes are the reverse of reactions occurring in many plants as a natural defence mechanism during the attack of herbivores. During the defence action, toxic HCN is released leaving the corresponding carbonyl compound (further described in Chapter 2 or^{48, 49}). The potential of hydrocyanation reactions was recognized and applied in many industrial processes, e.g. in the synthesis of previously described (*R*)-2-chloromandelic acid, mandelic acid (in both enantiomeric forms, described in section 1.3) and (*S*)-*m*-phenoxybenzaldehyde cyanohydrin. The latter is produced by DSM and Nippon Shokubai in a biphasic process using *Manihot esculenta* HnL and *Hevea brasiliensis* HnL overexpressed in *E. coli* and *Pichia pastoris* respectively. (*S*)-*m*-Phenoxybenzaldehyde cyanohydrin is an intermediate for the production of pyrethroids (insecticides).¹⁹ Yet another interesting example of industrial enzymatic hydrocyanation is stereoselective HCN addition to an α, β-unsaturated aldehyde, catalyzed by a recombinant HnL from *Hevea brasiliensis*, and simultaneous acylation to the corresponding acetylated cyanohydrin. A palladium-mediated chirality transfer reaction provides a precursor to coriolic acid (Figure 8).⁵⁰



Optically active α -hydroxy acids can be obtained via enantioselective reduction of water soluble α -ketocarboxylic esters with mutated *Saccharomyces cerevisiae* yeast or α -ketocarboxylic acids with e.g. *Staphylococcus epidermidis* D-dehydrogenase.^{47, 52} Two processes that apply dehydrogenases were developed independently by Degussa and Pfizer. Degussa patented a continuous process in which dehydrogenases from e.g. *Candida boidinii* or *Pseudomonas oxalaticus*, in a membrane bioreactor and with continuous regeneration of the NADH cofactor, are used for synthesizing e.g. lactic acid, (S)- and (R)-3-phenyl-2-hydroxypropionic acid, (S)- and (R)-2-hydroxy-4-methylvaleric acid, (S)- and (R)-2-hydroxy-3-methylbutyric acid or (S)- and (R)-2-hydroxyvaleric acid.^{53, 54}

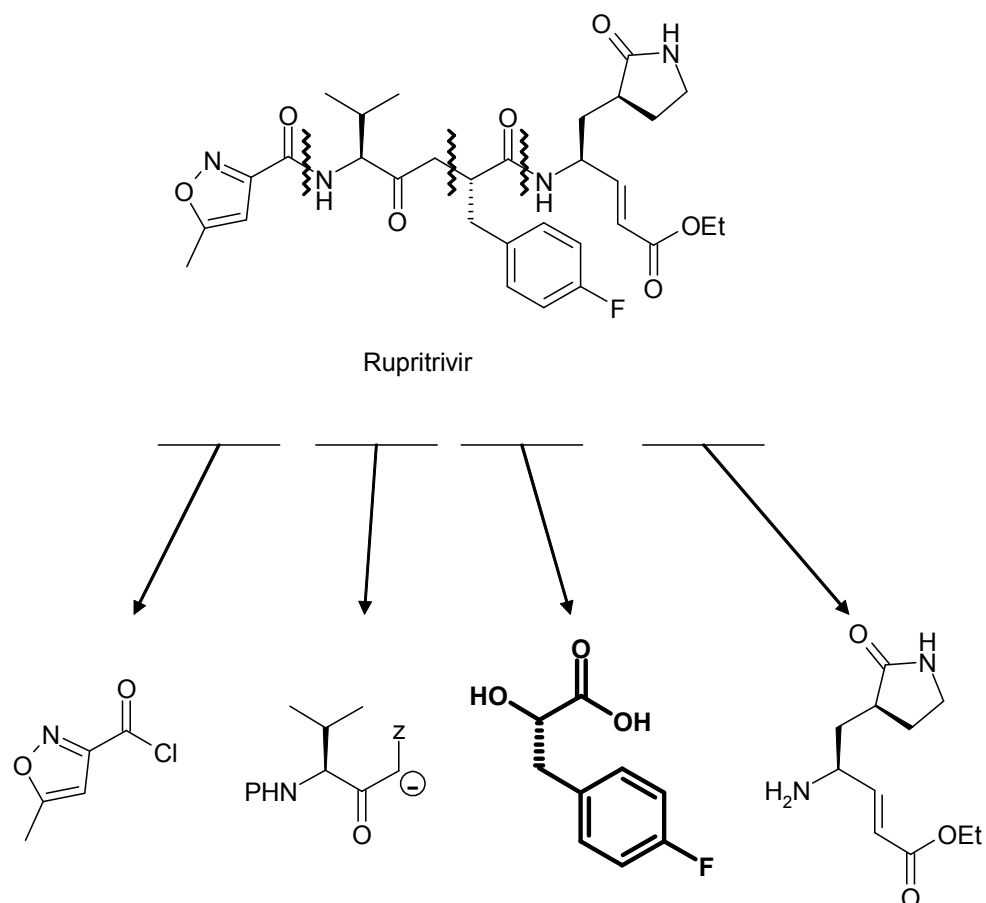


Figure 9. The building blocks of Rupintrivir.

Pfizer uses a similar process's process to produce (*R*)-3-(4-fluorophenyl)-2-hydroxypropionic acid, a building block of Rupintrivir (Figure 9). Pfizer's process (Figure 10) uses a D-dehydrogenase from *Leuconostoc mesenteroides* or *Staphylococcus epidermidis* at a multi-kilogram scale with a high space-time yield ($560 \text{ g} \cdot \text{l}^{-1} \cdot \text{d}^{-1}$), ee's of > 99% and overall yield of 68-72%. The starting material for (*R*)-3-(4-fluorophenyl)-2-hydroxypropionic acid synthesis is prepared in a separate step from 4-fluorobenzaldehyde and hydantoin.⁵⁵ Using the Pfizer process, other enantiopure α -hydroxy acids (in both enantiomeric forms) can also be synthesized.^{55,}

⁵⁶

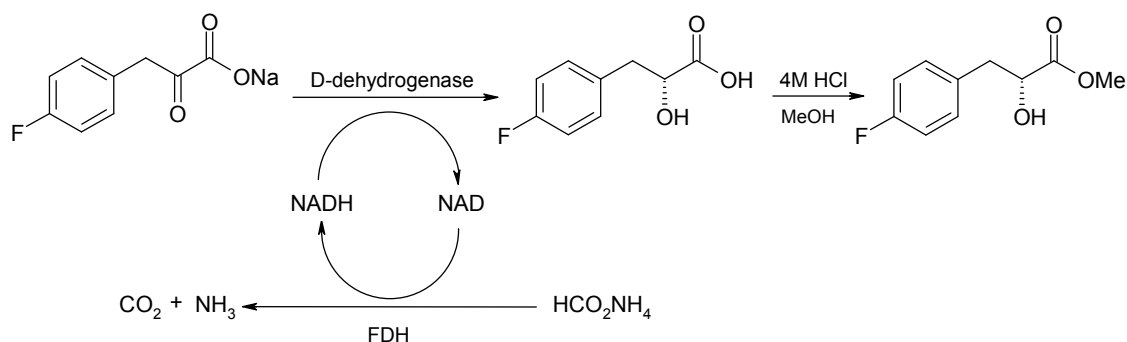


Figure 10. Pfizer's aqueous enzymatic and continuous reduction of 4-fluoro- α -ketophenylpropionic acid using (R)-lactate dehydrogenase (D-LDH) and formate dehydrogenase (FDH).

(S)- and (R)-lactic acid have traditionally been produced by fermentation with lactic acid bacteria.⁵⁷ Cargill developed and commercialized a fermentation process for (S)-lactic acid, which is converted into poly-lactic acid. Despite good stoichiometric yields, the latter process suffers from laborious downstream processing.⁵⁸

1.2.3. Stereoselective hydration of a prochiral compound

Another example of the enzymatic approach to enantiomerically pure α -hydroxy acids is hydration. Tanabe Seiyaku, for example, produces (S)-malic acid by enantioselective hydration of prochiral fumaric acid using immobilized fumarase originating from *Brevibacterium flavum* (Figure 11). Originally, this industrial process used *Brevibacterium ammoniagenes* but this was quickly substituted (in 1977) by *B. flavum* (immobilized with carrageenan) and applied in a continuous production process giving ~70% yield of the theoretical value.^{7, 59}

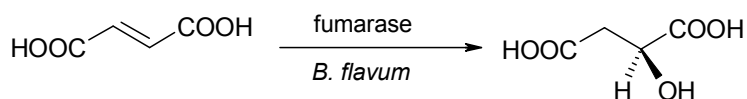


Figure 11. Tanebe Seiyaku production of (S)-malic acid through the enantioselective addition of water to fumaric acid.

1.3. Racemate resolution

Lipases play the major role in the kinetic resolution of racemic hydroxy acids. Enzymes such as CAL-A and lipase P are applied in the kinetic resolution of (*R,S*)-2-chloromandelic acid and (*R,S*)-2-hydroxyhexadecanoic acid, resulting in enantiomerically pure reaction products.^{11, 33} In another resolution method that uses penicillin amidase, enantiomerically pure α -hydroxy acids can be obtained from selective hydrolysis of the ester group of racemic α -hydroxy esters such as *O*-protected or (*R,S*)-mandelic acid methyl esters. The process with penicillin amidase is giving access to enantio-enriched (*S*)-hydroxy acids and with mandelic acid gives only moderate results (ee up to 40%).³ Another interesting method is a two-step chemo-enzymatic transesterification - enzymatic hydrolysis process. In this process aromatic *O*-acetylated chiral cyanohydrins are obtained from hydrolysis with an ester hydrolase (*Pseudomonas* sp.). The method allows reaching good chemical yields of the acetates (usually > 40%) and high enantiopurities (ee(*R*) > 98%). The resulting chiral *O*-acetylated cyanohydrin can be easily chemically hydrolyzed to the corresponding α -hydroxy acid (Figure 12).³

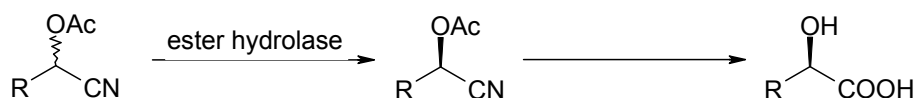


Figure 12. Two-step chemo-enzymatic transesterification - enzymatic hydrolysis process for the synthesis of enantiopure aromatic α -hydroxy acids.

Yet another resolution process that affords enantiopure α -hydroxy acids is the hydrolysis (dehalogenation) of α -chloro acids mediated by dehalogenases (enzymes with both, (*R*)- and (*S*)-selectivity are available) (Figure 13). ICI, Unitka and Astra Zeneca commercialized these processes (Astra Zeneca: 2000 ton·y⁻¹) to produce enantiopure (*S*)-lactic acid and (*S*)-2-chloropropionic acid with a (*R*)-dehalogenase from *Pseudomonas* sp. The latter acid is a key intermediate in the synthesis of optically active herbicides.^{53, 57, 60}

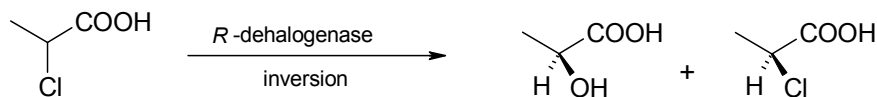


Figure 13. Preparation of (*S*)-2-chloropropionic acid using an (*R*)-specific dehalogenase.

BASF uses an alternative technology based on (*R*)-lactic acid fermentation and chemical steps to obtain the same molecule.⁶⁰

Enantioselective hydrolysis of the racemic cyanohydrin represents another attractive method for the preparation of chiral α -hydroxy acids. To achieve this Nature has developed two general pathways to degrade nitriles into carboxylic acids (Figure 14).^{61, 62} The mechanism of the first route employs nitrile hydratases (NHase), a metalloenzyme that hydrolyzes a wide range of aliphatic, arylaliphatic, and aromatic nitriles into the corresponding amides; since the organisms that produce NHases usually also produce amidases, these amides can be further converted into the carboxylic acids and ammonia.⁶³ The other, nitrilase (NLase) -mediated, enzymatic route leads directly from nitriles to the carboxylic acids and ammonia.

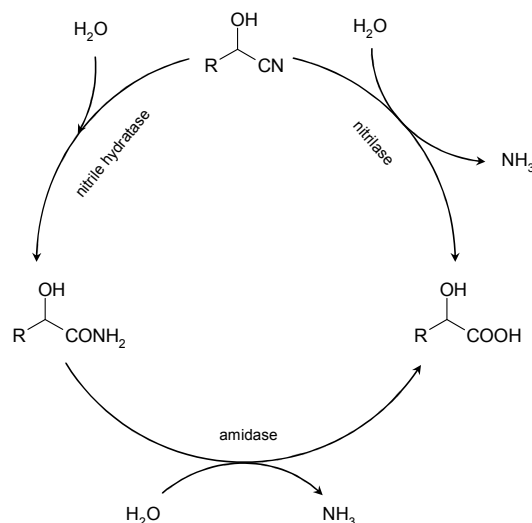


Figure 14. Two enzymatic pathways of the nitrile degradation into the corresponding hydroxy acid.

In one method, *O*-acylated mandelonitrile can undergo stereocontrolled biotransformation with *Rhodococcus* sp. AJ270 hydratase/ amidase giving enantiomerically enriched (*S*)-mandelic acid.³ In principle NHase/ amidase systems, in which nitrile hydrolysis occurs via the free amide (Figure 14), also provide access to chiral α -carboxylic acids.⁶⁴ However, only a small number of enantioselective nitrile

hydratases are known⁶⁵ and generally the enantiopurity is determined by an amidase that can be highly enantioselective towards α -substituted amides.⁶⁶ Due to the direct link of NLases with the thesis content, this class of enzymes will be treated proprietary and therefore will be described in more details in the following subchapter.

1.4. Nitrilases in syntheses of α -hydroxy acids

Almost 55 years ago Stowe *et al.* described a new type of enzyme that could cleave a nitrile, leaving the C – C bond untouched.⁶⁷ It was one of first publications on NLases. In the experiment Stowe and co-workers observed the direct conversion of indoleacetonitrile to indoleacetic acid by partially-purified *Avena coleoptiles* NLase.⁶⁷ Since the latter publication, and especially since the last 20 years, NLases have been gaining considerable attention. Passing the stages from discovery and lab curiosity, they have become significant industrial enzymes and are the subject of a number of recent reviews.⁶⁸⁻⁷⁴ All known NLases share a highly conserved region of amino acid sequence which includes a cysteine that is responsible for the catalytic activity of the enzyme.⁷⁴ All NLases discovered until now are either (*R*)- or non-enantioselective with respect to α -hydroxy acids.^{75, 76} The selectivity can be used in the kinetic resolution of e.g. (*R,S*)-mandelonitrile in the presence of *Alcaligenes faecalis* NLase to give (*R*)-mandelic acid.⁸ The prominent examples of NLase application in industrial scale synthesis are the asymmetric, chemo-enzymatic processes from BASF and Mitsubishi Rayon (Figure 15, route B).^{73, 77}

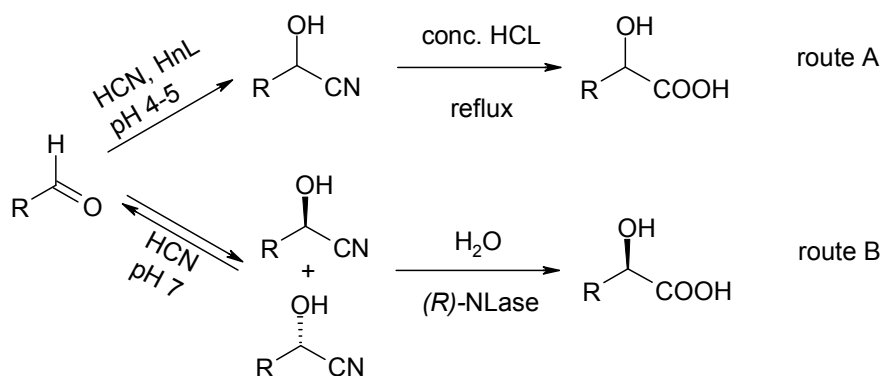


Figure 15. Synthetic routes to enantiomerically pure α -hydroxy acids, via HnL catalyzed enantioselective hydrocyanation (route A) and (R) -NLase mediated dynamic kinetic resolution (route B).

Mitsubishi Rayon and BASF have commercialised a process in which enantiopure α -hydroxy acids can be synthesized *via* a dynamic kinetic resolution (DKR) of the (chemically synthesised) cyanohydrin in the presence of an enantioselective NLase (*Alcaligenes faecalis* ATCC 8750⁷⁸ and *E. coli* JM109³ respectively). Racemisation of the cyanohydrin *via* reversible de-hydrocyanation, takes place readily at pH 7 or above. The latter methodology is attractive on account of the mild reaction conditions and is industrially applied in the multi-ton scale synthesis of (R) -mandelic acid.^{73, 77} Route A (Figure 15) on the other hand, was developed by DSM and is based on the enantioselective hydrocyanation of the appropriate aldehyde in the presence of an HnL which gives rise to the corresponding enantiomerically pure cyanohydrin, followed by chemical hydrolysis in the presence of strong acid (process described in detail earlier in the Chapter). This latter step generates copious quantities of salt and is not compatible with sensitive functional groups, which is a serious limitation.

In a more classical approach, enantiopure (R) - or (S) -mandelic acid can also be obtained using non-selective NLases such as *Rhodococcus rhodochrous* IFO 15564 or *P. fluorescens*. Such NLases hydrolyse both enantiomers of (R,S) -mandelonitrile. To obtain the pure enantiomers a resolution of the cyanohydrin before the enzymatic hydrolysis is needed.⁷⁹

The synthesis of α -hydroxy acids using NLases, such as presented in the previous paragraph and the DKR methodology depend not only on the availability of highly enantioselective biocatalysts but also require an enzyme that will generate a minimum amount of amide. This latter issue may seem trivial and has long been

disregarded somewhat, but reports of modest amounts of amide coproducts date back to the early days of NLase enzymology. Only recently has the subject gained more attention^{62, 75, 80} and it was demonstrated that it has a relationship with the stereochemistry of the nitrile.^{62, 75}

The enzymatic routes in which α -hydroxy acids are the products and in which the starting material is a cyanohydrin, like in enzymatic hydrolysis of nitriles, where either NLases or a tandem of nitrile hydratase and amidase is used bears several advantages over the chemical routes. The enzymatic processes take place at mild reaction conditions and with high chemo-, regio-, and stereoselectivity, leading to high purity products and energy savings due to the absence of byproducts, salts and metal wastes.⁷³

2. Objective of the thesis and survey of its contents

The work described in this thesis deals with sustainable, one-pot multienzyme routes to synthesize enantiomerically pure α -hydroxy acids. To achieve this goal, different approaches using HnL and NLases or HnL, NLases and nitrile hydratases (NHases) were studied. The enzymes in the bioconversions were used in the form of single- or combi-CLEA (double or triple) (Cross Linked Enzyme Aggregate). The latter three-enzyme immobilizate was developed in the course of the present investigations and was extensively studied for optimum immobilization conditions as well as immobilizate operational and storage stability. An alternative approach was also studied and compared to the corresponding combi-CLEA. In this system, the enzymes were co-expressed in one *E. coli* cell. Because of the NLase side-activity, the mechanism of *P. fluorescens* NLase in relation to NHase activity was studied. Looking for new enzymes with cyanohydrin hydrolyzing properties, the project led to the discovery of a new NLase. The enzyme was characterized for its biocatalytic properties. In Chapter 8 we also describe the discovery of unique properties of some surfactants acting as hydrocyanation catalysts. Using these in combination with lipases, we attempted to design a chemo-enzymatic system towards the synthesis of enantiomerically pure, protected cyanohydrins.

3. Reference list

1. Kastiris, E.; Dimopoulos, M. A. *Best Pract. Res. Cl. Ha.* **2007**, *20*, 681-699.
2. Moorlag H. *Chiral α -Substituted α -Hydroxy Acids*; Rijksuniversiteit Groningen: 1991.
3. Gröger, H. *Adv. Synth. Catal.* **2001**, *343*, 547-558.
4. Shai, A.; Maibach, H. I.; Baran, R. *Handbook of Cosmetic Skin Care; α -Hydroxy Acids*. Informa Health Care: 2001: pp. 187-196.
5. Coppola, G. M.; Schuster, H. F. *α -Hydroxy Acids in Enantioselective Syntheses*; VCH: 1997.
6. Sokoll, K. K., Chong, P., and Klein, M. H. Biodegradable Copolymer Containing α -Hydroxy Acid and α -Amino Acid Units. [US 6042820]. 1996.
7. Breuer, M.; Ditrach, K.; Habicher, T.; Hauer, B.; Kessler, M.; Stürmer, R.; Zelinski, T. *Angew. Chem., Int. Ed.* **2004**, *43*, 788-824.
8. Yamamoto, K.; Oishi, K.; Fujimatsu, I.; Komatsu, K. I. *Appl. Environ. Microbiol.* **1991**, *57*, 3028-3032.
9. Goodman, L. S.; Gilman, A. *The Pharmacological Basis of Therapeutics*; MACMILLAN: 1970; pp. 1042.
10. Tao, J.; Zhao, F. *Supplement to Chimica Oggi/Chemistry Today* **2006**, *24*, 6-7.
11. Uhm, K. N.; Lee, S. J.; Kim, H. K.; Kang, H. Y.; Lee, Y. J. *Mol. Catal. B: Enzym.* **2007**, *45*, 34-38.
12. Wang, P. Y.; Chen, T. L.; Tsai, S. W. *Enzyme Microb. Technol.* **2006**, *39*, 930-935.
13. Ema, T.; Okita, N.; Ide, S.; Sakai, T. *Org. Biomol. Chem.* **2007**, *5*, 1175-1176.
14. Li, J. J.; Johnson, D. S.; Sliskovic, D. R.; Roth, B. D. *Contemporary Drug Synthesis*; Wiley-Interscience: 2004. pp. 1-10.
15. Kleemann, A. *Ullmann's Encyclopedia of Industrial Chemistry* **2008**; pp.28
16. Bousquet, A. and Musolino, A. Hydroxyacetic Ester Derivatives, Preparation Method and Use as Synthesis Intermediates. [US 6573381]. 2003.
17. Bousquet, A. and Musolino, A. Hydroxyacetic Ester Derivatives, Preparation Method and Use as Synthesis Intermediates. [US 6894186]. 2005.
18. Hwang, S. O., Kim, K. S., Young, J., and Jung, Ch. O. Method for Preparing Clopidogrel and its Derivatives. [WO 2009/151256].
19. Liu, Z.; Weis, R.; Glieder, A. *Food Technol. Biotechnol.* **2004**, *42*, 237-249.

20. Poechlauer, P.; Skranc, W.; Wubbolts, M. In *Asymmetric Catalysis on Industrial Scale: Challenges, Approaches and Solutions*; Blaser, H. U.; Schmith, E. Eds. The Large-Scale Biocatalytic Synthesis of Enantiopure Cyanohydrins. Wille-VCH: 2003; pp. 151-163.
21. Maddin, S. *Skin Therapy Letter* **1998**, 3, 1-6.
22. van Scott, E. J.; Yu, R. J. *Arch. Dermatol.* **1974**, 110, 586-590.
23. Seager, S. L.; Slabaugh, M. R. *Chemistry for Today: General, Organic and Biochemistry*; Thomson Books/Cole: 2004. pp.476-478.
24. Nicoletti, I.; Corradini, C.; Cogliandro, E.; Cavazza, A. *Int. J. Cosmetic Sci.* **1999**, 21, 265-274.
25. Berardesca, E. In *Handbook of Cosmetic Science and Technology*; Barel, A. O.; Paye, M.; Maibach, H. Eds. α -Hydroxy Acids. Marcel Dekker: 2001; pp. 311-315.
26. Kakeya, H.; Sakai, N.; Sugai, T.; Ohta, H. *Agr. Biol. Chem. Tokyo* **1991**, 55, 1877-1881.
27. Brown, H. C.; Pai, G. G.; Jadhav, P. K. *J. Am. Chem. Soc.* **1984**, 106, 1531-1533.
28. Wynberg, H.; Staring, E. G. J. *J. Org. Chem.* **1985**, 50, 1977-1979.
29. Wynberg, H.; Staring, E. G. J. *J. Am. Chem. Soc.* **1982**, 104, 166-168.
30. Effenberger, F.; Heid, S. *Tetrahedron: Asymmetry* **1995**, 6, 2945-2952.
31. Effenberger, F.; Horsch, B.; Weingart, F.; Ziegler, T.; Kuhner, S. *Tetrahedron Lett.* **1991**, 32, 2605-2608.
32. Horn, D. H. S.; Pretorius, Y. Y. *J. Chem. Soc.* **1954**, 1460-1464.
33. Sugai, T.; Ohta, H. *Agr. Biol. Chem. Tokyo* **1990**, 54, 3337-3338.
34. Gamboni, R.; Mohr, P.; Waespesarcevic, N.; Tamm, C. *Tetrahedron Lett.* **1985**, 26, 203-206.
35. Davis, F. A.; Vishwakarma, L. C. *Tetrahedron Lett.* **1985**, 26, 3539-3542.
36. Evans, D. A.; Morrissey, M. M.; Dorow, R. L. *J. Am. Chem. Soc.* **1985**, 107, 4346-4348.
37. Prati, L.; Rossi, M. In *Green Chemistry. Challenging Perspectives*; Tundo, P.; Anastas, P. Eds. Selective catalytic oxidation of 1,2-diols in alkaline solution: an environmentally friendly alternative for α -hydroxy-acids production. OXFORD University Press: 2000; pp. 183-200.
38. Mikami, K.; Terada, M.; Nakai, T. *J. Am. Chem. Soc.* **1989**, 111, 1940-1941.
39. Helmchen, G.; Wierzchowski, R. *Angew. Chem., Int. Ed. Engl.* **1984**, 23, 60-61.
40. Ludwig, J. W.; Newcomb, M.; Bergbreiter, D. E. *Tetrahedron Lett.* **1986**, 27, 2731-2734.
41. Pearson, W. H.; Cheng, M. C. *J. Org. Chem.* **1986**, 51, 3746-3748.
42. Kolasa, T.; Miller, M. J. *J. Org. Chem.* **1987**, 52, 4978-4984.
43. Oppolzer, W.; Dudfield, P. *Helv. Chim. Acta* **1985**, 68, 216-219.

44. Coppola, G. M.; Schuster, H. F. In *Asymmetric Synthesis. Construction of Chiral Molecules Using Amino Acids*; Coppola, G. M.; Schuster, H. F. Eds. The Leucine Family. John Wiley & Sons, Inc.: 1987; pp. 109-126.
45. Lazarus, M.; Adam, W.; Saha-Möller, C. R.; Schreier, P. In *Oxygen and Life. Oxygenases, Oxidases and Lipid Mediators*; Ishimura, Y.; Nozaki, M.; Yamamoto, S.; Shimatzu, T.; Narumiya, S.; Mitani, F. Eds. Biotransformations with oxidases and peroxidases for the synthesis of optically active building blocs. Elsevier Health Sciences: 2002; pp. 45-49.
46. Adam, W.; Lazarus, M.; Saha-Möller, C. R.; Schreier, P. *Accounts Chem. Res.* **1999**, 32, 837-845.
47. Deol, B. S.; Ridley, D. D.; Simpson, G. W. *Aust. J. Chem.* **1976**, 29, 2459-2467.
48. Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, 348, 1655-1661.
49. Wajant, H.; Effenberger, F. *Biol. Chem.* **1996**, 377, 611-617.
50. Fessner, W.-D. *Curr. Opin. Chem. Biol.* **1998**, 2, 85-97.
51. Yadav J.S.; Deshpande, P. K.; Sharma G.V.M. *Pure Appl. Chem.* **1990**, 62, 1333-1338.
52. Kim, M. J.; Kim, J. Y. *J. Chem. Soc.-Chem. Comm.* **1991**, 326-327.
53. Sheldon, R. A. In *Chirotechnology. Industrial Synthesis of Optically Active Compounds*; Sheldon, R. A. Ed. Enzymatic Transformations. Marcel Dekker, Inc.: 1993; pp. 205-270.
54. Wandrey, C., Wichmann, R., Leuchtenberger, W., Kula, M. R., and Buckmann, A. Process for the Continuous Enzymatic Change of Water Soluble α -Ketocarboxylic Acids into the Corresponding α -Hydroxycarboxylic acids. [US 4326031]. 1982.
55. Tao, J.; McGee, K. *Org. Process Res. Dev.* **2002**, 6, 520-524.
56. Tao, J.; McGee, K. In *Asymmetric Catalysis on Industrial Scale*; Blaser, H. U.; Schmith, E. Eds. Development of an Efficient Synthesis of Chiral α -Hydroxy Acids. Wiley-vch: 2004; pp. 323-334.
57. Motosugi, K.; Esaki, N.; Soda, K. *Biotechnol. Bioeng.* **1984**, 26, 805-806.
58. Benninga, H. *A History of Lactic Acid Making*; Kluwer Academic Publishers: 1990.
59. Takata, I.; Yamamoto, K.; Tosa, T.; Chibata, I. *Enzyme Microb. Technol.* **1980**, 2, 30-36.
60. Schulze, B.; Wubbolts, M. G. *Curr. Opin. Biotech.* **1999**, 10, 609-615.
61. Sorokin, D. Y.; Pelt, S. v.; Tourova, T. P.; Muyzer, G. *Appl. Environ. Microbiol.* **2007**, 73, 5574-5579.
62. Fernandes, B. C. M.; Mateo, C.; Kiziak, C.; Chmura, A.; Wacker, J.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, 348, 2597-2603.

63. Kobayashi, M.; Shimizu, S. *Nat. Biotechnol.* **1998**, *16*, 733-736.
64. Kobayashi, M.; Shimizu, S. *Curr. Opin. Chem. Biol.* **2000**, *4*, 95-102.
65. Prepachalova, I.; Martinkova, L.; Stolz, A.; Ovesna, M.; Bezouska, K.; Kopecky, J.; Kren, V. *Appl. Microbiol. Biot.* **2001**, *55*, 150-156.
66. Hirrlinger, B.; Stolz, A.; Knackmuss, H. J. *J. Bacteriol.* **1996**, *178*, 3501-3507.
67. Stowe, B. B.; Thimann, K. V. *Arch. Biochem. Biophys.* **1954**, *51*, 499-516.
68. Martinková, L.; Mylerová, V. *Curr. Org. Chem.* **2003**, *7*, 1279-1295.
69. Martinková, L.; Křen, V. *Biocatal. Biotransform.* **2002**, *20*, 73-93.
70. O'Reilly, C.; Turner, P. D. *J. Appl. Microb.* **2003**, *95*, 1161-1174.
71. Banerjee, A.; Sharma, R.; Banerjee, U. C. *Appl. Microbiol. Biot.* **2002**, *60*, 33-44.
72. Brenner, C. *Curr. Opin. Struct. Biol.* **2002**, *12*, 775-782.
73. Wieser, M.; Nagasawa, T. In *Stereoselective Biocatalysis*; Patel, R. N. Ed. Stereoselective Nitrile-Converting Enzymes. Patel: 2000; pp. 461-486.
74. DiCosimo R. In *Biocatalysis in the Pharmaceutical and Biotechnology Industries*; R.N.Patel Ed. Nitrilases and Nitrile Hydratases. CRC Press: 2007; pp. 1-26.
75. Kiziak, C.; Conradt, D.; Stolz, A.; Mattes, R.; Klein, J. *Microbiology-Sgm* **2005**, *151*, 3639-3648.
76. Rustler, S.; Muller, A.; Windeisen, V.; Chmura, A.; Fernandes, B. C. M.; Kiziak, C.; Stolz, A. *Enzyme Microb. Technol.* **2007**, *40*, 598-606.
77. van Rantwijk, F.; Mateo, C.; Chmura, A.; Fernandes, B.; Sheldon, R. A. In *Modern Biocatalysis: Stereoselective and Environmentally Friendly Reactions*; Wolf-Dieter Fessner; Thorleif Anthonsen Eds. Nitrilases in the Enantioselective Synthesis of α -Hydroxycarboxylic Acids. Wiley-vch: 2009; pp. 261-272.
78. Endo, T., Yamagami, T. and Tamura, K. Biological Process for Producing α -Hydroxy Amide or α -Hydroxy Acid. Nitto Chemical Industry Co., Ltd. Tokyo Japan [US 532 6702]. 1994.
79. Sugai, T.; Yamazaki, T.; Yokoyama, M.; Ohta, H. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1419-1427.
80. Winkler, M.; Glieder, A.; Klempier, N. *Chem. Commun. (Cambridge)* **2006**, 1298-1300.

**Cross-Linked Enzyme Aggregates
of the Hydroxynitrile Lyase from *Manihot esculenta*:
Highly Active and Robust Biocatalyst**

2

A part of this Chapter contents have been published in:

Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, 348, 1655-1661.

1. Introduction

Release of hydrogen cyanide (HCN) (cyanogenesis) represents a widespread defence mechanism in over 3000 plants, many insects and some bacteria.^{1, 2} HCN in such plants is stored as α -hydroxynitriles which are stabilized by O- β -glycosidic linkage to saccharides. In the defence activated situations, the HCN-saccharide bond is broken and in course of the enzymatic cyanogenic reaction catalyzed by a hydroxynitrile lyase, HCN is released to defend the plant.³ In 1908 Rosenthaler adopted that part of the plant defence machinery for synthetic purposes. In his experiment he used an almond meal emulsion to synthesize (*R*)-mandelonitrile from benzaldehyde and HCN.⁴ Further experiments had to wait until the 1960's, however. Since then (*R*)- and (*S*)-selective hydroxynitrile lyases have gained ever growing popularity and eventually broke the line of being laboratory curiosities and became real industrial enzymes in organic synthesis.² The broad substrate tolerance compared with other (*S*)-oxynitrilases, and the high activity are directly reflected in the synthetic potential of the (*S*)-selective hydroxynitrile lyase from *Manihot esculenta* (MeHnL). When its potential was recognized the enzyme was cloned and over-expressed in *E. coli* and immobilized on cellulose and nitrocellulose to increase its stability.^{5, 6}

Enzyme stability under reaction conditions is always an issue and often the free enzyme, when exposed to reactants and reaction medium, will undergo inactivation. Stabilization of free MeHnL has been achieved by immobilizing the enzyme on cellulose, silica gel,⁷ alumina or clay.⁸ In one of the previous papers published from this laboratory, we described the successful preparation of a CLEA of the (*R*)-specific oxynitrilase from *Prunus amygdalus*. An indisputable advantage of CLEAs in comparison to carrier fixed preparations is that the former consist of nearly pure protein and, hence, exhibit high productivities.

A previous study of MeHnL precipitation and cross-linking revealed ammonium sulfate to be the most effective precipitation agent.⁹ The lyophilized MeHnL CLEA was selected for further tests where the immobilizate was used as the hydrocyanation catalyst in the reactions schematically shown in Figure 1.

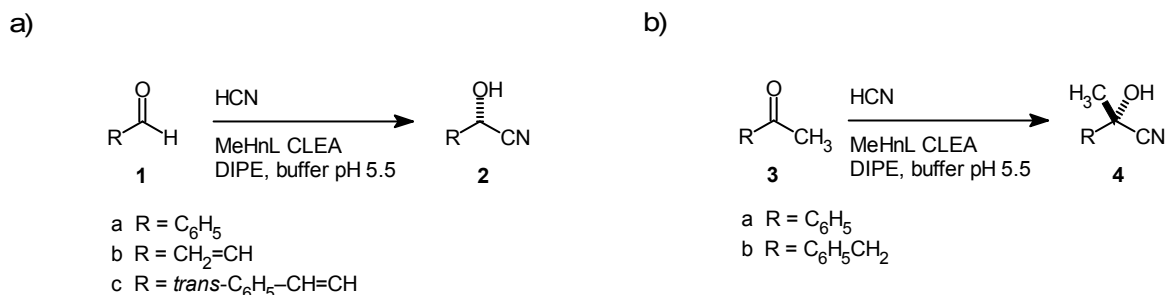


Figure 1. Enzymatic hydrocyanation of aldehydes (a) and ketones (b).

In this Chapter we will present a cross-linked enzyme aggregate (CLEA) of MeHnL. The immobilizate was tested in bi-phasic hydrocyanation reactions consisting of organic solvents with a palette of various aldehydes and ketones. Related work has recently been published from our laboratory.¹⁰

2. Results and discussion

2.1. Synthetic application in microaqueous medium

The enantioselective addition of hydrogen cyanide to aldehydes and ketones in the presence of enzymes is a very sensitive reaction and the enantiopurity of the product is greatly dependent on the water content and the pH.^{4, 11} Hence, the reaction is preferably carried out in non-aqueous medium such as diisopropyl ether (DIPE). The use of a water-immiscible organic solvent with a minimum water phase, in which the enzyme catalyzed reaction takes place, efficiently suppresses the spontaneous, uncatalyzed reaction.¹² Although reaction rates in biphasic aqueous-organic media are generally lower than in water, the isolated yields and enantiopurities are usually better.¹¹⁻¹³ The reactions described in the following part of this Chapter were carried out in diisopropyl ether (DIPE) containing a minimum quantity of buffer.

The first tested substrate, benzaldehyde (**1a**) was converted into the corresponding (*S*)-mandelonitrile (**2a**) in under 4 h (Figure 2). The initial product ee was, at 96%, rather low and further dropped in the course of the reaction. This latter effect is ascribed to the unselective, spontaneous hydrocyanation reaction that progresses

competitively to the enzymatic reaction in the test with 10% buffer (v/v). Additionally, ee drop of **2a** may be caused by non-enzymatic cycling of reactant and product.

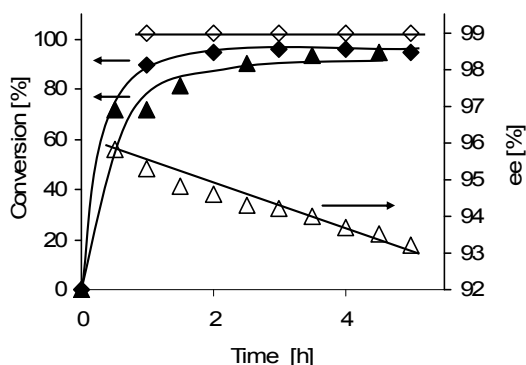


Figure 2. Hydrocyanation of **1a** (100 mM) in DIPE-citrate buffer pH 5.5 and 25 °C: MeHnL CLEA (22 MSU mL⁻¹), 10% buffer, 500 mM HCN, progress (▲), ee (△): MeHnL (7 MSU mL⁻¹), 2% buffer, 700 mM HCN, progress (◆), ee (◇).

A significant improvement in the product purity was observed when the same reaction was carried out with reduced buffer content (2% v/v, see Figure 2). In such low buffer content, all the aqueous phase was absorbed by the biocatalyst making the reaction monophasic and (*S*)-**2a** was obtained with 99% ee. Further experiments showed that the MeHnL CLEA maintains its activity in media containing only 0.2% (v/v) of buffer (Table 1). This extreme stability of MeHnL CLEA was confirmed in a related study.¹⁰

To prove the potential of our enzymatic method, the hydrocyanation of **1a** in DIPE containing 2% buffer was scaled up to 25 g scale. Cyanohydrin **2a** was obtained in nearly quantitative yield and > 99% ee.

Table 1. Hydrocyanation of aldehydes and ketones in the presence of MeHnL CLEA.

Reactant	Enzyme [MSU mL ⁻¹]	Buffer [%]	HCN [equivs.]	Time [h]	Conv. [%]	ee [%]
1a	22	10	5	4.0	94	94
	7	2.0	7	3.0	96	99
	7	0.2	7	5.5	95	99
1b	3	0.1	6	1.0	96	49
	9	0.0	5	1.0	96	42
*	12	0.1	12	2.0	99	55
1c	400	2.5	6	6.2	86	87
	400	2.5	12	6.2	90	92
3a	32	0.2	12	3.8	7	>99
	32	0.2	12	23	12	91
*	32	0.2	12	23	11	98
3b	17	0.5	12	2.5	90	>96

Reaction conditions: as described in the material and methods part.

* Reaction at 0 °C

Enantioselective, enzymatic hydrocyanation of 2-propenal (**1b**) into (S)-**2b** is generally considered troublesome. The best results reported in the literature with two very similar (S)-oxynitrilases, namely MeHnL and *Hevea brasiliensis* oxynitrilase, give dramatically different results in terms of enantiopurity: MeHnL afforded **2b** with 56% ee⁵ whereas almost enantiopure product (98% ee) resulted from hydrocyanation in the presence of the *H. brasiliensis* enzyme.¹² The differences in enantiopurity prompt the question whether it is related to the nature of the enzymes or caused by a nonenzymatic background reaction. The first bioconversion of **1b** in microaqueous DIPE showed a rapid progress of the hydrocyanation reaction; full conversion was not obtained, however, as only 96% conversion was reached with three-fold HCN excess. The enantiopurity was monitored and we found that the initial ee was 55 – 56% but dropped to under 40% when the reaction proceeded towards equilibrium (Figure 3). Separate experiments in which we lowered the reaction pH to 4 and further reduced the buffer content, showed that the moderate ee is not a result of a non-enzymatic background reaction (data not shown). Interestingly, MeHnL CLEA is active in water free DIPE (Table 1), but even then no beneficial effect on the product purity was observed. Similar observations were made in a parallel study.¹⁰

Significantly improved ee (55%) and conversion (> 99%) were obtained when the reaction was carried out at 0 °C. To achieve this, the enzyme loading was doubled to

compensate for the loss of activity caused by the lower reaction temperature and the HCN excess was increased to 12-fold to push the reaction towards total conversion of **1b** (Table 1).

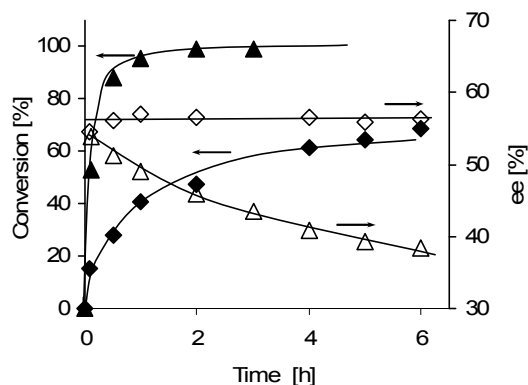


Figure 3. Hydrocyanation of **1b** (100 mM) in DIPE-citrate buffer pH 5.5 and 25 °C; MeHnL CLEA (3 MSU mL⁻¹), 0.1% buffer, 660 mM HCN, progress (▲), ee (△): MeHnL (1.8 MSU mL⁻¹), 500 mM HCN, progress (◆), ee (◇).

Yet another aldehyde, cinnamaldehyde (**1c**) is a slow-reacting substrate with MeHnL and additionally suffers from an unfavorable equilibrium.¹⁴ The best literature result for converting **1c** into **2c** is 80% conversion and 95% ee.¹⁵ We tried to equal these results with experiments where we used increased amount of enzyme (Table 1), but only 84% conversion with 87% ee was obtained. Better results however, were obtained when the HCN excess was increased to twelve-fold and **2c** was obtained in 90% yield and 93% ee.

Figure 4 shows an experiment in which **1c** is converted into **2c** with a reduced amount of the enzyme to prolong the reaction over time. The analytical measurements showed no background reaction. The enantioselectivity of the enzyme is high, but is not sustained and already at < 50% conversion the erosion of the ee of **2c** is observed.

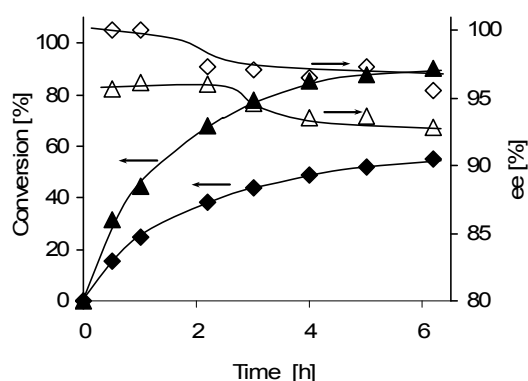


Figure 4. Hydrocyanation of *trans*-**1c** (100 mM) in DIPE-citrate buffer pH 5.5 and 25 °C; MeHnL CLEA (400 MSU mL⁻¹), 0.25% buffer, 1.2 M HCN, progress (▲), ee (△): MeHnL (200 MSU mL⁻¹), 500 mM HCN, progress (◆), ee (◇).

Acetophenone (**3a**) is known to be a difficult substrate for hydrocyanation, due to the unfavorable ketone – cyanohydrin equilibrium. When the reaction is catalyzed with MeHnL in biphasic medium, the ee of the product (**4a**) is usually also modest and the reported values do not exceed 87%.^{5, 15} Hydrocyanation tests with **3a** performed in a bi-phasic reaction medium consisting of 0.2% DIPE and with different reactant concentrations revealed the reaction not to progress beyond 8 – 12% (Table 1). We checked if the poor yield is caused by inactivation of the enzyme. Adding extra portions of MeHnL did not have any effect, indicating that equilibrium had indeed been reached. The initial high enantiopurity of (*S*)-**4a** (> 99%) decreased while approaching the equilibrium. This decrease in ee was not caused by non-enzymatic hydrocyanation as the latter was negligible (approx. 1% after 24h). A better ee was obtained when the reaction was performed at 0 °C. 98% Pure (*S*)-**4a** was obtained, however at only 11% **3a** conversion (Table 1).

The last tested ketone, 1-phenylacetone (**3b**) was much more reactive than **3a** and 90% conversion was reached already after 2.5h with ee > 96% (*S*)-**4b** (Table 1).

The question arises whether the low product ee is caused by non-enzymatic background reactions or by MeHnL-catalyzed reactions. The transformations described in the previous paragraphs were all carried out in media with very much reduced water contents. Under such conditions, we found non-enzymatic reactions to be insignificant. Therefore we conclude that the enantiopurity decrease observed in reactions with **1a – c** and **3a** while approaching equilibrium is most likely caused by

enzymatic dehydrocyanation of (preferentially) the (*S*)-product, which will cause any (*R*)-product to accumulate.

3. Conclusions

The previously optimized MeHnL CLEA was successfully applied in hydrocyanations of different aldehydes and ketones. The immobilizate proved to be extremely stable under the reaction conditions resisting high reactant concentrations and extensive DIPE content. The enzyme is active even in pure organic solvent where no buffer is present.

In most cases, using our microaqueous bi-phasic approach, we obtained products with higher enantiopurities than those reported in literature. We also found that the decay of the product ee observed upon the approach of the reaction equilibrium was caused by enzymatic cycling of reactant and product.

4. Materials and methods

Chemicals

Semi-purified (*S*)-hydroxynitrile lyase from *Manihot esculenta* (3000 U·mL⁻¹) was obtained from Jülich Fine Chemicals (Jülich, Germany). A CLEA of MeHnL (batch no. CLEAMEHNL-S03-150-03512, 78 mandelonitrile synthetic units (MSU see below) mg⁻¹) from CLEA Technologies (Delft, The Netherlands) was used in the synthetic hydrocyanation reactions.

Acetophenone, acrolein, benzaldehyde, cinnamic aldehyde, diisopropylether, 1,3-dimethoxybenzene and 1-phenyl-2-propanone were obtained from Acros, Aldrich or Fluka and were used without further purification. (*R,S*)-mandelonitrile was obtained from Fluka. (*R,S*)-2-hydroxy-3-butenenitrile,¹⁶ 2-hydroxy-2-methyl-3-phenylpropionitrile¹⁴ and 2-hydroxy-4-phenyl-*trans*-3-butenenitrile¹⁴ were prepared according to literature procedures.

A 2 M solution of hydrogen cyanide in DIPE was prepared from sodium cyanide as described.¹⁷ **Warning:** sodium cyanide and HCN are highly poisonous. They should be handled in a well working fume hood and a HCN detector should be present at all times.

Analytical methods

The progress of the reactions was measured by HPLC; enantiomeric purities were measured by chiral HPLC or GC as described. HPLC analyses were carried out on Waters 510 pump and a Waters 468 variable wavelength UV detector at 215 nm. GC analyses were performed on a Shimatzu GC-17 instrument equipped with a FID detector and a Varian Inc. 25 m x 0.32 mm Chirasil-Dex CB column; the carrier gas was N₂. Samples (10 µL) were treated with a derivatization reagent (300 µL) that contained acetic anhydride (0.8 mL) and pyridine (0.8 mL) in dichloromethane (10 mL) and analyzed after standing for 1h. Further details are given below.

Kinetic activity assay

2-Hydroxy-4-phenyl-*trans*-3-butenitrile: To citrate buffer pH 5.5 (2 mL, 20 mM) were added a MeHnL solution (250 µL) or an equivalent amount of CLEA, DIPE (0.4 mL), cinnamic aldehyde solution in DIPE (2 M, 0.4 mL) and HCN solution in DIPE (2 M, 1.2 mL). The mixture was stirred and the temperature of 0 °C was maintained. The reaction vessel was kept closed to prevent HCN escape.

A 20 µL sample was taken every h from the organic phase for up to 3 h and dissolved in hexane containing 2% TFA (1 mL). The remaining water was removed by adding some Na₂SO₄. The sample was centrifuged and analyzed by HPLC.

Mandelonitrile: to citrate buffer pH 5.5 (2 mL) in a 10 mL screw cap vessel were added an appropriate amount of enzyme preparation, HCN solution in DIPE (2 M, 1.2 mL) and DIPE (700 µL). The mixture was stirred magnetically, chilled to 0 °C in ice/water and benzaldehyde was added (100 µL). After 10 min (< 30% conversion) a sample (10 µL) was taken from the organic phase, diluted with eluent (990 µL), dried over Na₂SO₄ and analyzed by HPLC. One mandelonitrile synthesis unit (MSU) will synthesize one µmol of mandelonitrile per min under these conditions.

Enzymatic hydrocyanation: general procedure

The reagents and internal standard (1,3-dimethoxybenzene) were taken from 2 M stock solutions in DIPE. DIPE was kept saturated with 200 mM citrate buffer pH 5.5 (unless noted otherwise). The MeHnL CLEA catalyst was kept as a suspension in DIPE and appropriate amounts were pipetted into the reaction mixtures.

The hydrocyanation reactions were carried out in 2 mL glass reactors equipped with a PTFE sealed screw cap. The appropriate amounts of buffer, DIPE, MeHnL CLEA suspension, HCN solution and internal standard were introduced into the reactor, which was magnetically stirred and thermostated using a water bath (25 °C) or a cryostat (0 °C). The reaction was started by adding the aldehyde or ketone (100 mM with respect to the total volume)

Samples (10 µL) for HPLC analysis were taken periodically, diluted with eluent (990 µL). Dried over Na₂SO₄ and centrifuged prior to injection.

Benzaldehyde: The stock solution of benzaldehyde was kept over saturated NaHCO₃ to prevent accumulation of benzoic acid. The hydrocyanation reactions of benzaldehyde were carried as described in the general procedure. Progress and ee were measured by HPLC (4.6 x 250 mm 5 µ Chiracel OB-H column (Daicel), eluent hexane: 2-propanol (90:10, v/v) at 0.5 mL·min⁻¹ at 35 °C).

Acrolein: The hydrocyanation reactions of acrolein were carried as described in the general procedure. The reaction progress was measured by HPLC (4.6 x 250 mm 5 µ Chiracel OB-H column (Daicel), eluent hexane:2-propanol (90:10, v/v) at 0.5 mL/min). Chiral analysis was carried out by GC as described above: temperature program: 10 min at 100 °C, then a gradient of 13 °C min⁻¹ to 180 °C.

Cinnamic aldehyde: The hydrocyanation reactions of cinnamic aldehyde were carried as described in the general procedure. The reaction progress was measured by HPLC (4.6 x 250 mm 5 µ Chiracel OB-H column (Daicel), eluent hexane:2-propanol (90:10, v/v) at 0.5 mL·min⁻¹). Chiral analysis was carried out by GC as described above at 160 °C.

Acetophenone: The hydrocyanation reactions of acetophenone were carried as described in the general procedure. The reaction progress was measured by HPLC (4.6 x 250 mm 5 µ Chiracel OB-H column (Daicel), eluent hexane:2-propanol (90:10, v/v) at 0.5 mL·min⁻¹ at 35 °C).

1-Phenylpropanone: The hydrocyanation reactions of 1-phenylpropanone were carried as described in the general procedure but at 6 mL scale in 10 mL reaction vessel to accommodate the increased sample volume. The reaction progress was measured by HPLC (4.6 x 250 mm 5 µ Chiracel OD-H column (Daicel), eluent hexane:2-propanol (90:10, v/v) at 0.5 mL·min⁻¹).

Samples for chiral analysis (200 μ L) were mixed with a derivatization reagent composed of butyric anhydride (40 μ L), *N,N*-dimethylaminopyridine (40 mg) in dioxane (400 μ L), stirred for 2 h and washed with dilute HCl. A sample of the organic phase was taken, diluted with eluent and analyzed by HPLC (4.6 x 250 mm 5 μ Chiracel OB-H column (Daicel), eluent hexane:2-propanol (90:10, v/v) at 0.5 mL·min⁻¹).

Large-scale synthesis of (S)-mandelonitrile

To DIPE (0.25 L) in a 1 L round-bottomed flask were added MeHnL CLEA (0.25 g, 19.6 kMSU) and 20 mM citrate buffer pH 5.5 (2 mL). The mixture was stirred vigorously in an ice-water bath until a fine suspension was obtained. HCN was added as a 2 M solution in DIPE (200 mL), followed by a solution of benzaldehyde (25 g, 0.24 mol) in DIPE (250 mL). The mixture was stirred at room temperature.

The progress and ee were monitored as described above. Complete conversion was obtained in 2h. The reaction mixture was filtered to remove the biocatalyst, dried over Na₂SO₄ and concentrated under vacuum at 50 °C until the last traces of HCN had been removed. (S)-mandelonitrile was obtained as colourless oil in 99% yield and 99.6% ee.

5. Reference list

1. Effenberger, F. *Angew. Chem., Int. Ed. Engl.* **1994**, 33, 1555-1564.
2. Gregory, R. J. H. *Chem. Rev.* **1999**, 99, 3649-3682.
3. Wajant, H.; Effenberger, F. *Biol. Chem.* **1996**, 377, 611-617.
4. Schmidt, M.; Griengl, H. In *Biocatalysis- From Discovery to Application*; Fessner, W.-D. Ed. Oxynitrilases: From Cyanogenesis to Asymmetric Synthesis. Springer: 1999; pp. 194-226.
5. Forster, S.; Roos, J.; Effenberger, F.; Wajant, H.; Sprauer, A. *Angew. Chem., Int. Ed. Engl.* **1996**, 35, 437-439.
6. North, M. *Tetrahedron: Asymmetry* **2003**, 14, 147-176.
7. Semba, H.; Dobashi, Y.; Matsui, T. *Biosci., Biotechnol., Biochem.* **2008**, 72, 1457-1463.
8. Semba, H. and Dobashi, Y. Immobilized Euphorbiaceae, Poaceae or Olacaceae S-Hydroxynitrile Lyase. 09/758,317 [US 6709847 B2]. 2004.

9. Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1655-1661.
10. Cabirol, F. L.; Hanefeld, U.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1645-1654.
11. Effenberger, F.; Ziegler, T.; Forster, S. *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 458-460.
12. Griengl, H.; Klempier, N.; Pochlauer, P.; Schmidt, M.; Shi, N. Y.; Zabelinskaja-Mackova, A. A. *Tetrahedron* **1998**, *54*, 14477-14486.
13. Griengl, H.; Hickel, A.; Johnson, D. V.; Kratky, C.; Schmidt, M.; Schwab, H. *Chem. Commun.* **1997**, 1933-1940.
14. Gerrits, P. J.; Willeman, W. F.; Straathof, A. J. J.; Heijnen, J. J.; Brussee, J.; van der Gen, A. *J. Mol. Catal. B: Enzym.* **2001**, *15*, 111-121.
15. Buhler, H.; Effenberger, F.; Forster, S.; Roos, J.; Wajant, H. *Chembiochem.* **2003**, *4*, 211-216.
16. Gassman, P. G.; Talley, J. J. *Tetrahedron Lett.* **1978**, 3773-3776.
17. van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Org. Process Res. Dev.* **2004**, *7*, 828-831.

Cross-Linked Enzyme Aggregates of Two Enzymes (combi-CLEA)

3

A part of the Chapter contents have been published in:

Mateo, C.; Chmura, A.; Rustler, S.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Tetrahedron: Asymmetry* **2006**, 17, 320-323.

1. Introduction

In the introductory Chapter (Chapter 1), two chemo-enzymatic pathways are described that are industrially used in the synthesis of enantiopure α -hydroxy acids. One is based on enantioselective hydrocyanation of the appropriate aldehyde in the presence of an oxynitrilase (HnL, E.C. 4.1.2.10), which gives rise to the corresponding enantiomerically pure cyanohydrin, followed by chemical hydrolysis in the presence of a strong acid (Figure 1A). This latter step generates copious quantities of salt and is not compatible with sensitive functional groups, which is a serious drawback. Alternatively, enantiopure α -hydroxy acids can be obtained via a dynamic kinetic resolution of the (chemically synthesized) cyanohydrin in the presence of an enantioselective nitrilase (NLase, E.C. 3.5.5.1, see Figure 1B). This latter methodology, which is industrially applied in the multi-ton-scale synthesis of (*R*)-mandelic acid,¹⁻³ is restricted to (*R*)- α -hydroxy acids, because no NLases that preferentially hydrolyse (*S*)-cyanohydrins have been identified until now.

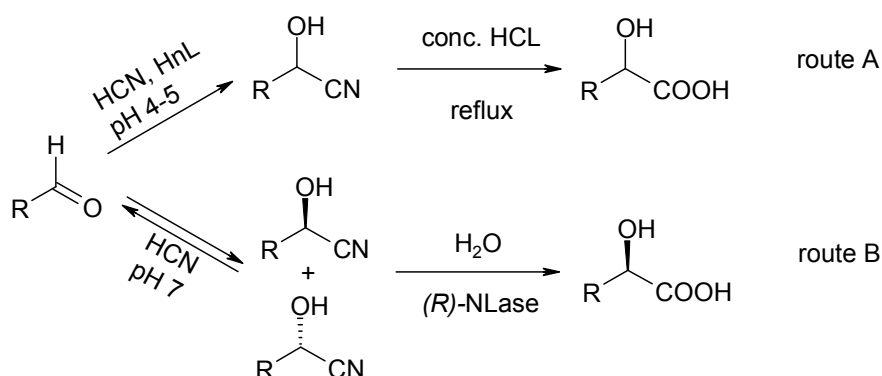


Figure 1. Synthetic routes to enantiomerically pure α -hydroxy acids, via HnL catalyzed enantioselective hydrocyanation (route A) and (*R*)-NLase mediated dynamic kinetic resolution (route B).

We surmised that a fully enzymatic route to the (*S*)-hydroxy acids should be possible by combining an (*S*)-selective HnL and a non-selective NLase in a bienzymatic cascade (Figure 2). Besides being more environmentally acceptable, the mild reaction conditions of the combined enzymatic reaction are compatible with a wide range of hydrolysable groups.

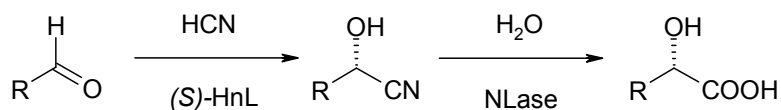


Figure 2. Bienzymatic procedure for the synthesis of (S)-2-hydroxy acids, using an (S)-specific HnL and a non-specific NLase in tandem.

The use of two different enzymes in one reaction can potentially cause incompatibilities. The first issues that should be resolved are the pH and the reaction medium. HnL-mediated hydrocyanations are preferably carried out at $\text{pH} < 5$ to suppress the competing uncatalyzed hydrocyanation. This background reaction is usually further reduced by the use of a biphasic aqueous-organic or micro-aqueous reaction medium (described in detail in Chapter 2). Such reaction conditions are tolerated rather well by the readily available (S)-selective HnL from *M. esculenta* (MeHnL).⁴ NLases, in contrast, have a pH optimum at 7 – 8 and are readily deactivated by organic solvents. Rendering the NLase compatible with the conditions of enzymatic hydrocyanation is obviously an issue on which the success of the methodology depends. It was reasoned that immobilization as a cross-linked enzyme aggregate (CLEA)⁵⁻⁷ would present a solution to this latter problem. This Chapter introduces the concept of the co-immobilization of MeHnL and a NLase, as a combi-CLEA. Besides, it presents the application of the combi-CLEA bienzymatic procedure as well as a comparison with the system containing two separate CLEAs in the synthesis of enantiomerically pure (S)-mandelic acid. Furthermore, an attempt at a one-pot synthesis of 2-ethylmandelic acid (atrolactic acid) is described. The Chapter also addresses the problems related to the activity recovery of the combi-CLEA, its storage stability and recyclability. Finally the reactant inhibition effects on the co-immobilized enzymes are reported.

2. Results and discussion

2.1. Activity recovery in the combi-CLEA

Immobilization of enzymes usually results in a partial loss of their activity. It is thought that in CLEAs this can be caused by chemical or/and conformational changes in the active sites or their direct surroundings. In order to gain more insight in the

immobilization we desired to assay the activities of the enzymes in the combi-CLEAs separately. Suitable assay protocols were developed and described in detail in Chapter 4. Results of activity recovery measurements in a combi-CLEA composed of MeHnL and *Pseudomonas fluorescens* EBC191 NLase (PfNLase) and cross-linked with dextran polyaldehyde showed that cross-linking with dextran polyaldehyde resulted in high activity recoveries; 57% for MeHnL and 90% for the NLase. No activity was found in the crosslinking supernatant.

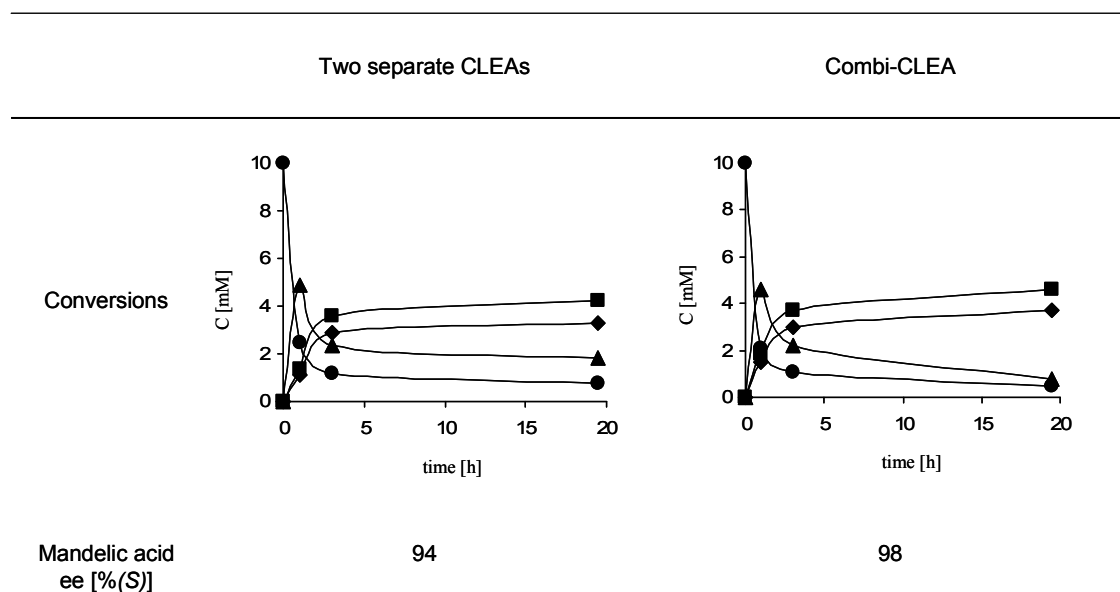
A similar experiment was performed with *Prunus amygdalus* (*R*)-HnL and with the same NLase as above. For the aggregation and cross-linking, similar conditions were used as in the above experiment. The activity recovery measurements showed that much less activity was found in this co-immobilizate compared to the combi-CLEA with MeHnL. The recovered activity was 30% for (*R*)-HnL, and only 13% for PfNLase. Despite a very similar cross-linking procedure, recovery of PfNLase in the co-immobilizates dropped from excellent (90%) in the combi-CLEA with MeHnL to only 13% in the combi-CLEA with (*R*)-HnL. No activity was found in the cross-linking supernatant. The quite unexpected differences in PfNLase activity recoveries in these two experiments can possibly be ascribed to cross-interactions of (*R*)-HnL and PfNLase or impurities or adjuvants in the semi-purified enzyme solutions. Mixing and cross-linking PfNLase with HnL of different origin could cause different e.g. coating effect on PfNLase, or more possibly, different HnLs are stored in different solutions and one of them strongly influences PfNLase eventually leading to its poor activity recovery.

2.2. Applications of the combi-CLEA in the bienzymatic transformations

2.2.1. Synthesis of (*S*)-mandelic acid. Comparison of the bienzymatic synthesis with two CLEAs and a combi-CLEA. Influence of pH

Prior to the main experiments, the enzymatic hydrolysis of mandelonitrile (**1**) was investigated in some detail, as this is evidently the critical step. It should be fast, moreover, to avoid accumulation – and possible racemisation – of (*S*)-**1**. Few NLases displayed a useful activity at pH 5.5 – 6; most of these were strongly (> 90% ee) biased towards the (*R*)-enantiomer and converted (*S*)-**1** only quite sluggishly, if at all

(data not presented). The previously mentioned one, a recombinantly expressed PfNLase,⁸ was an exception as it converted (*S*)- and (*R*)-**1** at comparable rates at pH 5.5,⁸ which was adopted as a compromise pH for the bienzymatic reactions. The experiments with CLEAs of MeHnL and the chosen NLase in tandem were conducted in a 90:10 diisopropyl ether (DIPE)-buffer pH 5.5 biphasic medium (Figure 3).



Reaction conditions: Buffer:DIPE 10:90, 10 mM benzaldehyde (**2**), 50 mM HCN, 25 °C

Figure 3. Bienzymatic synthesis of (*S*)-mandelic acid (**3**) from **2** and HCN in the presence of MeHnL and PfNLase. Comparison of two CLEAs and combi-CLEA. **2** (●), **1** (▲), (*S*)-**3** (■), (*S*)-mandelic amide (**4**, ◆).

The reaction with two CLEAs proceeded to nearly full conversion and the product ee was 94%. Combining both enzymes in a bienzymatic catalyst (combi-CLEA) resulted in further improvement and 98% enantiomerically pure (*S*)-**3** was obtained. It would seem that the nitrile intermediate is immediately hydrolysed in the combi-CLEA particles, which suppresses diffusion into the water phase and possible racemisation. A full analysis of the reaction products showed, however, that very appreciable amounts of (*S*)-**4** (approx. 40%) accompanied the formation of the main product. Finally, we attempted to improve the reaction rate by increasing the pH to 6, in the expectation that the faster *in-situ* hydrolysis of (*S*)-**1** would prevent its racemisation.

The increase in rate was quite modest, however, and came at the expense of a slight, 2% decrease in product ee (96%) (data not shown).

In conclusion, the formation of nearly enantiomerically pure (*S*)-**3** proves that the bienzymatic methodology is basically sound and that racemisation of the intermediate can be avoided. The formation of large amounts of (*S*)-**4** is an obstacle that will be addressed later. Also, the subject of amide formation was separately investigated and presented in Chapter 6.

2.2.2. Synthesis of atrolactic acid

In principle, using the bienzymatic procedure, it should be possible to fully convert aldehydes and ketones with an unfavorable hydrocyanation equilibrium into the corresponding α -hydroxy acids. This is, because the co-immobilized NLase should consistently convert the formed cyanohydrin into the corresponding acid, which prevents the first reaction from reaching equilibrium. Acetophenone was chosen as a model substrate (Figure 4).

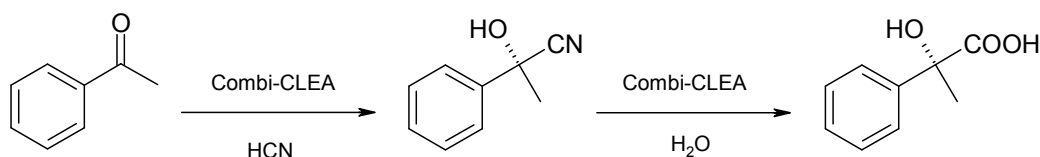


Figure 4. One pot, bienzymatic synthesis of (*S*)-atrolactic acid.

Earlier experiments showed that the hydrocyanation of acetophenone did not proceed beyond 12% conversion (14-fold HCN excess, 1.5% buffer biphasic system, see Chapter 2). The present, bienzymatic conversions of acetophenone with the combi-CLEA were carried out in biphasic system with 50% buffer of pH 5.5 and 10-fold HCN excess (Table 1).

Table 1. Bienzymatic synthesis of atrolactic acid in 50% DIPE biphasic system.

Time [h]	Acetophenone [mM]	Atrolactic acid [mM]	
0	9.1	0	
1	9.0	0.02	
5	8.6	0.1	
23.5	8.3	0.3	
96	8.2	0.3	
140	8.0	0.3	Combi-CLEA extra portion
7 days	8.0	0.4	
20 days	8.0	0.4	

Reaction conditions: Buffer:DIPE 50:50, 10 mM acetophenone, 100 mM HCN, 25 °C

The goal of the reaction was not achieved. The reaction stopped after converting 12% acetophenone from which 30% was further converted into atrolactic acid. Presumably, the rest remained in the reaction medium as 2-hydroxy-2-phenylpropionitrile (this compound could not be detected by the analytical method used). After addition of an extra portion of the combi-CLEA, further reaction progress was observed. Hence, it would seem that the biocatalyst had become inactive. As shown further in this Chapter, indeed, some chemicals such as HCN showed a deactivating effect on the combi-CLEA. Unfortunately, the separate PfnLase deactivation studies with 2-hydroxy-2-phenylpropionitrile were not possible due to nitrile instability in the reaction medium.

2.3. Effect of the reactant concentration

One goal of this PhD project was to construct a stable and robust multi-enzyme catalyst that would effectively catalyze the synthesis of enantiopure α -hydroxy acids. One of the parameters that determine the usefulness of a catalyst in large scale syntheses is its operational stability in the presence of elevated reactant concentrations (all the previous experiments with the combi-CLEA were carried out at 10 mM concentration). Increased reactant concentrations may negatively influence (immobilized) enzymes, causing their partial or total deactivation. Rustler *et al.*⁹ have

already reported that free PfNLase cleared extract is sensitive to elevated concentrations of reactants.

In the following batch experiments, it was checked if the co-immobilized MeHnL and PfNLase are able to handle higher **2** starting concentrations. Five experiments (10 – 250 mM) (Table 2) were conducted with the same amount of the combi-CLEA. The tests were carried out in a biphasic system with 30% buffer of pH 5.5.

Table 2. Bienzymatic syntheses of (S)-**3** starting with different **2** concentrations in biphasic system with 30% buffer of pH 5.5.

2 [mM]	HCN excess	Time [h]	2 [mM]	1 [mM]	4 [mM]	3 [mM]	3 ee [% (S)]
10	5	1.6	<1	0	4	6	n.d.
		20	0	0	4	6	99
25	5	1.6	0	<1	10	14	n.d.
		20	0	0	10	14	99
42	5	1.6	<1	<1	18	23	n.d.
		20	0	0	18	23	>96
83	5	1.6	0	8	40	36	n.d.
		20	0	>7	40	36	>96
250	3	2	0	<1	155	111	96
		27	0	0	153	110	96

n.d. – not determined

Reaction conditions: buffer: DIPE 30:70, 25 °C

In general, the tests were successful, showing that over the whole range of concentrations **2** was completely converted in less than 2 h. Also, PfNLase showed good activity, converting all (S)-**1** formed into the corresponding (S)-**3** and (S)-**4** within the same time scope. The enantiopurity of the synthesized (S)-**3** was in 10 – 83 mM reactions >96%, and in 250 mM reaction, 96% (after 27 h). A surprising observation was made when comparing the ratios of **4** and **3** formed in the course of the above reactions (Figure 5). As a general trend, an increasing bias towards amide formation with the HCN concentration was observed (from 0.6 amide/acid in 50 mM HCN to 1.4 in the reaction with 750 mM HCN present). A pH shift towards acid cannot be the cause as none was observed; moreover HCN is too weak (pKa 9.31)

to shift a citrate buffer at pH 5.5. As it will be discussed later, the phenomenon rather seems to be related to the general inhibition of PfNLase by HCN.

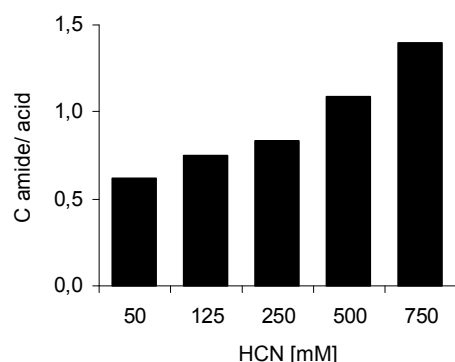


Figure 5. The effects of the HCN starting concentration on the amide/acid ratio.

2.4. Inhibition of the EBC191 NLase in the combi-CLEA by the reactants

2.4.1. Inhibition by benzaldehyde

It was decided to investigate the possible inhibition or deactivation of PfNLase under the reaction conditions in more detail. Thus, a MeHnL/PfNLase combi-CLEA was exposed to **2** at 10 – 500 mM concentration. After 1 h, phenylacetonitrile was added to the mix and the NLase activity was assayed (Figure 6). No dramatic effect of **2** on the activity of PfNLase was found. The activity of the enzyme decreased by 10% when incubated in 10 mM **2**. The activity loss increased somewhat at higher **2** concentrations and seems to level off at 20%.

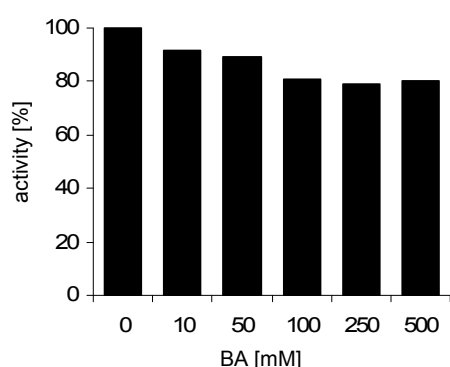


Figure 6. Inhibition of PfNLase in the combi-CLEA by **2**.

2.4.2. Inhibition by HCN

HCN inhibition experiments, analogous to the ones described above, were carried out in biphasic 70% aqueous DIPE at pH 5.5. The combi-CLEA was incubated with HCN (25 – 575 mM) for 1 h. Subsequently, phenylacetonitrile was added to the reaction mixture and the NLase activity was assayed (Figure 7).

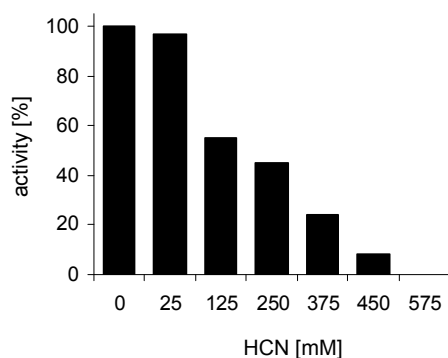


Figure 7. Residual activity of PfNLase upon 1 h exposure to HCN.

At low (25 mM) HCN concentration the activity loss was insignificant, but exposure to 0.12 M HCN already caused the NLase activity to drop to 55%. Upon increasing the concentration to 0.58 M, a total and irreversible loss of activity was observed. In the following experiments the combi-CLEA was incubated with 0.12 M HCN. Samples of the combi-CLEA were withdrawn from the aqueous phase and PfNLase activity was assayed (Figure 8a). For comparison, an identical experiment with free PfNLase was carried out (Figure 8b).

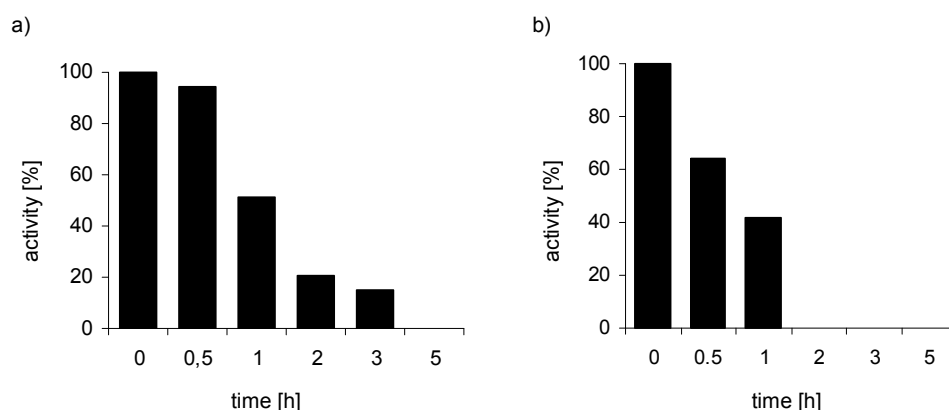


Figure 8. PfNLase activity loss during the enzyme incubation with 0.12 M HCN. Combi-CLEA (a), free PfNLase (b).

A fairly rapid (and irreversible) loss of activity was observed in these experiments. The free enzyme lost activity much faster (5% and 36% loss after 0.5 h for the combi-CLEA and the free enzyme respectively). No deactivation effect was observed in the control reaction where no HCN was present. The half-life time for PfNLase in the combi-CLEA was 65 min while for the free NLase it was 51 min. Presumably, the PfNLase undergoes a structural change when exposed to HCN, which seems to be slower in the CLEA. Yet another possibility is that the PfNLase could be inhibited by ammonium formate that possibly could be formed by NLase catalyzed hydrolysis of HCN.

Separate tests showed that the MeHnL activity was not affected by HCN (data not shown).

2.5. Storage stability of the combi-CLEA

Possible advantages of enzyme immobilization are improved storage stability as well as enhanced operational stability in the presence of organic solvents and easy recyclability.^{10, 11} A stability test was performed by preparing a combi-CLEA batch of MeHnL and PfNLase which was resuspended in citrate buffer pH 5.5 and stored on ice. The residual activities of the enzymes were assayed periodically over 13 days (Figure 9).

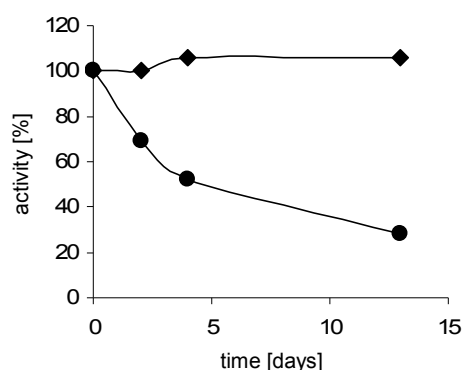


Figure 9. Combi-CLEA stability on ice in 20 mM citrate buffer pH 5.5. MeHnL (◆), NLase (●).

Surprisingly, the storage in citrate buffer at 0 °C resulted in a significant loss of the NLase activity over time. Already after 2 days, the enzyme had lost 25% of its initial activity. The activity loss continued reaching 72% after 13 days. MeHnL, in contrast,

was not affected. Experiments described in the following Chapter (Chapter 4) showed that the deactivation of the NLase in this kind of immobilizate is not caused by oxidation of the catalytic cysteine.¹² It was also shown that the NLase co-immobilizate stability can be greatly improved by storing the biocatalyst in a saturated salt solution (see next Chapter).

2.6. Recycling stability of the combi-CLEA

In a recycling stability test, the standard combi-CLEA conversions of **2** in a biphasic system were performed. After every reaction, the combi-CLEA was washed two times with citrate buffer and used in the next cycle. MeHnL and PfNLase activities were measured by monitoring the reaction progress. Changes in activities of both enzymes are shown in Figure 10.

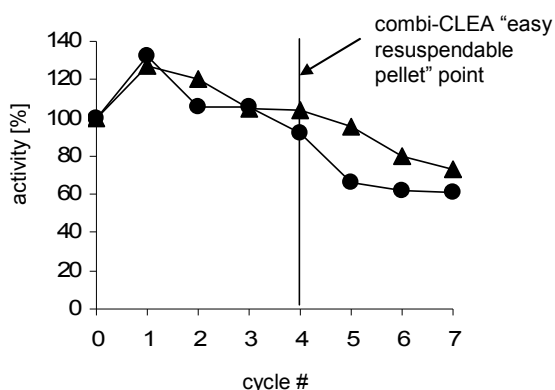


Figure 10. The recyclability of the combi-CLEA. MeHnL (▲), PfNLase (●).

In total, during one day, seven 30 min reactions and reconditioning cycles were carried out. During this time, in all seven tests almost all **2** was converted into (S)-**3** and (S)-**4** (data not shown). A consistent drop in reaction rate was observed starting from cycle no 1. After the seventh, cycle the rate of the reaction was 30% lower than the initial value. Considering the excellent stability of the MeHnL CLEA observed in other experiments, we tentatively concluded that the observed activity loss was mainly a result of mechanical losses occurring during transfer of the CLEA in the recycling steps and not to deactivation of PfNLase by HCN. This is supported by the observation that the physical characteristics of the CLEA changed and it became difficult to spin down after the fourth cycle and mechanical losses result if decantation

is not done very carefully. This corresponded with the point at which activity losses started.

3. Conclusions

We successfully prepared a co-immobilizate of MeHnL and PfNLase in the form of a combi-CLEA. Under the cross-linking conditions applied the activity recovery was high for both enzymes. The biphasic experiments with **2** showed the formation of nearly enantiomerically pure (*S*)-**3** demonstrating that the bienzymatic methodology is basically sound and that racemisation of the intermediate can be avoided. Furthermore, the combi-CLEA gave better results in terms of enantiopurity than in reactions with two separate CLEAs. The unexpected formation of large amounts of (*S*)-**4** was observed. However, the combi-CLEA can be applied in reactions with up to 0.25 M **2**. The operational stability of the combi-CLEA is however effected, by HCN that causes PfNLase deactivation. Finally, the experimental data showed that the combi-CLEA has excellent recyclability properties. The storage stability test revealed that unlike MeHnL, PfNLase is not stable while stored in citrate buffer on ice.

4. Materials and methods

Chemicals

Semi-purified (*S*)-hydroxynitrile lyase from *M. esculenta* [E.C. 4.1.2.10] (protein content 88 mg mL⁻¹) and *Prunus amygdalus* (*R*)-hydroxynitrile lyase were obtained from Jülich Fine Chemicals (Jülich, Germany). *E. coli* JM109 heterologously expressed EBC191 NLase from *Pseudomonas fluorescens* [E.C. 3.5.5.1] was obtained as described.⁸ The NLase cell-free extract contained 34 mg protein mL⁻¹. Phenylacetoneitrile and phenylacetic acid were obtained from MERCK-Schuchardt, (*R,S*)-mandelic amide 97% from Alfa Aesar. *Trans*-cinnamaldehyde +99%, benzaldehyde (redistilled), (*R,S*)-mandelic acid and *trans*-cinnamaldehyde +99% from Acros Organics. (*R,S*)-Atrolactic acid hemihydrate (98%), benzoic acid, Veratrole +99% and DIPE were purchased from Fluka. All the chemicals were used as provided by suppliers, without further purification. Dextran polyaldehyde: was

obtained by oxidation of Dextran 100-200 kDa (Serva Feinbiochemica) as described.⁶ HCN: was prepared as previously described.⁴ 2-hydroxy-4-phenyl-trans-3-butenitrile (modified procedure¹³): 0.02 mol of freshly distilled *trans*-cinnamaldehyde was reacted overnight in 0.1 M phosphate buffer pH 7.5 with 0.6 mol HCN (2 M solution in DIPE) (added dropwise) in a magnetically stirred reactor. When the reaction reached equilibrium (> 90% conversion), the cyanohydrin was extracted into (50:50) CH₂Cl₂/hexane and dried over MgSO₄. Solvent was evaporated in vacuo until crystallization occurred spontaneously. The crystals were filtered off and dried. Fine, yellowish crystals were obtained. The structure of the product was confirmed by ¹H NMR.

Instrumentation

The reaction progress of all of the reactions was followed by HPLC – using Waters 590 pump and Waters 486 Tunable Absorbance Detector (alternatively, Alliance Waters 2695 Separation Module coupled with Waters 2487 Dual λ Absorbance Detector). The detector was set at 215 nm. The separations were achieved with 4.6 x 50 mm Merck ChromolithTM SpeedROD RP-18e column and H₂O:ACN (95:5) + 0.03% (v/v) heptafluorobutyric acid and 30 mM ammonium formate pH 2.3 as eluent (1 mL·min⁻¹).

The enantiomeric purity of (*R,S*)-mandelonitrile and the corresponding hydrolysis products were measured on HPLC system equipped with Waters 515 pump and Waters 486 Tunable Absorbance Detector, set at 215 nm. The separation was achieved with the straight phase 4.6 x 250 mm Chiralpak AD-H column and hexane:isopropanol (80:20) + 0.1% (v/v) TFA as eluent.

Protein content measurements

The total protein concentration of cell-free extracts were assayed according to the standard Bradford procedure.¹⁴ The samples were measured on a Shimadzu UV-240IPC spectrophotometer.

Free enzyme kinetic assays

One unit (U) of an enzyme will convert 1 μ mol of a substrate per min at 25 °C.

Oxynitrilase: Into 1.5 mL eppendorf filled up with 0.5 mL 20 mM citrate buffer pH 5.5, enzyme solution (0.3 mg), 395 μ L DIPE, IS (veratrole) and HCN (100 mM) were added. Finally, *trans*-cinnamaldehyde (10 mM) was added. The reaction was

incubated on the shaker at 25 °C. The reaction vessel was kept closed to prevent the escape of HCN. Samples were taken from each phase and analyzed on reversed-phase HPLC.

Nitrilase: 1.5 mL eppendorf was filled with 980 µL 20 mM citrate buffer pH 5.5, 10 µL (0.003 mg protein) 100x prediluted nitrilase solution, IS (veratrole) and finally phenylacetonitrile (10 mM) was added. Samples were taken and analyzed on reversed-phase HPLC.

Activity recovery in the combi-CLEA

MeHnL:PfNLase combi-CLEA: MeHnL (8.8 mg) and semi-purified PfNLase (3 mg) were mixed with dextran polyaldehyde solution (0.5 mL, cross-linking agent), 0.5 M phosphate buffer pH 7.5 (0.25 mL) and 1,2-dimethoxyethane (1 mL, aggregation agent). The mixture was stirred overnight at 4 °C and centrifuged. The pellet was resuspended in 0.1 M sodium bicarbonate solution containing 1 g·L⁻¹ of sodium borohydride (40 mL) to reduce the Schiff's bases, stirred for 45 min at 40 °C, washed three times with Milli-Q water (6 mL), resuspended in 20 mM citrate buffer pH 5.5 and stored at 4 °C.

(R)-HnL:PfNLase combi-CLEA: (R)-HnL (0.1 mg) and semi-purified PfNLase (3 mg) were mixed with dextran polyaldehyde solution (0.7 mL, cross-linking agent), 0.5 M phosphate buffer pH 7.5 (0.25 mL) and 1,2-dimethoxyethane (0.8 mL, aggregation agent). The mixture was stirred overnight at 4 °C and centrifuged. The pellet was resuspended in 0.1 M sodium bicarbonate solution containing 1 g·L⁻¹ of sodium borohydride (40 mL) to reduce the Schiff's bases, stirred for 45 min at 40 °C, washed three times with Milli-Q water (6 mL), resuspended in 20 mM citrate buffer pH 5.5 and stored at 4 °C.

Combi-CLEA activity recovery assays: The assays were carried out in identical way to those for the free enzymes assays (regarding the substrates and reaction conditions).

Table 3. The activity recovered in the combi-CLEA with MeHnL and PfNLase.

	Reaction rate [mg min ⁻¹ mg ⁻¹]	
	Free enzyme	combi-CLEA
MeHnL	0.41	0.23
PfNLase	13.5	12.2

Table 4. The activity recovered in the combi-CLEA with (*R*)-HnL and PfNLase.

	Reaction rate [mg min ⁻¹ mg ⁻¹]	
	Free enzyme	combi-CLEA
(<i>R</i>)-HnL	0.37	0.11
PfNLase	12.9	1.7

Storage stability of the combi-CLEA

Combi-CLEA preparation and storage: as described for MeHnL:PfNLase combi-CLEA. Quantities used: MeHnL (176 mg), PfNLase (204 mg), dextran polyaldehyde (10 mL), phosph. buffer (5 mL), 1,2-dimethoxyethane (0.03 mL). The batch was resuspended in 20 mM citrate buffer pH 5.5 and stored on ice. Samples for both HnL and NLase were taken periodically to check for the activity.

Activity assays: As described in “combi-CLEA activity recovery assays”.

One pot synthesis of (*S*)-mandelic acid

Combi-CLEA preparation: MeHnL and semi-purified PfNLase were mixed with dextran polyaldehyde solution (4 mL, cross-linking agent), 0.5 M phosphate buffer pH 7.5 (2 mL) and 1,2-dimethoxyethane (8 mL, aggregation agent). The mixture was stirred overnight at 40 °C and centrifuged. The pellet was resuspended in 0.1 M sodium bicarbonate solution containing 1 g L⁻¹ of sodium borohydride (40 mL) to reduce the Schiff's bases, stirred for 45 min at 4 °C, washed twice with Milli-Q water (40 mL), resuspended in 5 mL 0.5 M citrate buffer pH 5.5 and stored at 4 °C.

Reactions: Two CLEAs and in a separate reaction, the combi-CLEA suspension (0.5 mL), DIPE, HCN solution in DIPE (50 mM) were combined and DIPE was added to a total volume of 5 mL. benzaldehyde (10 mM) was added and the mixture was stirred vigorously at 25 °C. Samples were withdrawn periodically to check the progress of the reactions. For quantitative measurements 2 µL of the lower (aqueous) phase and 20 µL of organic phase were mixed with acidified mobile phase. DIPE was evaporated under vacuum, the sample spun down, and analyzed on reverse-phase HPLC (method described in the instrumentation part). The samples for enantiomeric purity measurements were first extracted from the aqueous phase to ethyl acetate and then measured on the straight phase column (method described in the instrumentation section).

Synthesis of atrolactic acid

0.1 mL combi-CLEA suspension (0.39 U MeHnL, 5.4 U PfNLase), 1.9 mL 20 mM citrate buffer pH 5.5, DIPE, HCN (100 mM) were combined and DIPE was added to a total volume of 4 mL (biphasic reaction with 50% DIPE). Acetophenone (10 mM) was added and the mixture was incubated on a shaker at 25 °C. Samples were withdrawn periodically from both phases and analyzed on reverse-phase HPLC (method described in the instrumentation section). After 140 h another combi-CLEA suspension portion was added (0.1 mL). HCN presence in the reaction was checked occasionally using an HCN detector.

The reactant concentration influence on the combi-CLEA

Combi-CLEA preparation: As described in “One pot synthesis of (S)-mandelic acid” experimental part.

Reactions: The conversions were carried out in 10 mL glass reactors equipped with magnetic stirrers and incubated at 25 °C, controlled by a thermostat. Into all the reactors, 1.0 mL of the fresh combi-CLEA suspension in 20 mM citrate buffer pH 5.5 and 1 mL of the same buffer were added. Then, a volume of DIPE, IS (benzoic acid) and a proper amount of benzaldehyde to obtain 10, 25, 42, 83 and 250 mM and HCN conc. 50, 125, 210, 415 and 750 mM respectively. The final reaction volumes were 6 mL and consisted of 70% organic phase. Samples were withdrawn periodically to check the progress of the reactions. For quantitative measurements 20 µL of the lower (aqueous) phase and 100 µL of organic phase were mixed with acidified mobile phase. DIPE was evaporated under vacuum, and analyzed on reverse-phase HPLC (method described in the instrumentation section). The samples for enantiomeric purity measurements were extracted from the aqueous phase to ethyl acetate and then measured on the straight phase column (method described in the instrumentation section).

Inhibition experiments

Benzaldehyde inhibition experiments: The tests were carried out in 1.5 mL eppendorf tubes and on Q.Instruments ThermoTWISTER comfort shaker at 25 °C. Into the tubes filled with 280 µL 20 mM citrate buffer pH 5.5, 20 µL combi-CLEA suspension was added (2.8 U NLase). DIPE and benzaldehyde (10 ÷ 500 mM) were added next. The organic phase was 70% of the total volume. After 1 h IS (veratrole)

and phenylacetonitrile were added to start the NLase activity assay. Samples were withdrawn periodically from both phases and analyzed on reverse-phase HPLC (method described in the instrumentation section).

HCN inhibition experiments: The tests were carried out in 1.5 mL eppendorf tubes and on Q.Instruments ThermoTWISTER comfort shaker at 25 °C. Into the tubes filled with 250 µL 20 mM citrate buffer pH 5.5, 50 µL combi-CLEA suspension was added (7.0 U NLase). DIPE and HCN (25 ÷ 575 mM) were added next. The organic phase was 70% of the total volume. After 1 h IS (veratrole) and phenylacetonitrile were added to start the NLase activity assay. Samples were withdrawn periodically from both phases and analyzed on reverse-phase HPLC (method described in the instrumentation section).

Incubation in 0.125 M HCN: The tests were carried out in 1.5 mL eppendorf tubes on Q.Instruments ThermoTWISTER comfort shaker at 25 °C. The stock, inhibition experiment was prepared in a 5 mL glass vial. In the vial the combi-CLEA was suspended in 20 mM citrate buffer pH 5.5 and DIPE (70 % v/v). To check the deactivation of the enzyme, samples from the aqueous phase of the stock solution (the combi-CLEA suspension) were withdrawn and transferred into eppendorfs filled with 920 µL 20 mM citrate buffer pH 5.5, IS (veratrole) and phenylacetonitrile (10 mM) were added. Samples were withdrawn periodically and analyzed on reverse-phase HPLC (method described in the instrumentation section).

Recycling stability of the combi-CLEA

Combi-CLEA preparation: as described for “Storage stability of the combi-CLEA” experiments.

Recycling experiments: The tests were carried out in 1.5 mL eppendorf tubes on Q.Instruments ThermoTWISTER comfort shaker at 25 °C. Into the tubes filled with 450 µL 20 mM citrate buffer pH 5.5, 50 µL portions of the combi-CLEA suspension were added (0.24 U MeHnL and 10.1 U PfNLase). DIPE (465 µL), IS (veratrole), HCN (50 mM) and benzaldehyde (10 mM) were added. The final reaction volume was 1 mL and consisted of 50% organic phase. After 30 min, the reaction was spun down at 10k rpm for 3 min, washed two times with citrate buffer and then the combi-CLEA was returned to the next reaction cycle. During the reaction, two samples were withdrawn. One after 5 min from the reaction start – to determine the enzymes

activities, and the other sample was taken after 30 min to check the total progress of the reaction. For quantitative measurements 10 μ L of each phase were mixed with 980 μ L of acidified mobile phase and analyzed on reverse-phase HPLC (method described in the instrumentation section except the eluent. Here, H₂O:ACN (90:10) + 0.1% (v/v) TFA was used as the mobile phase).

5. Reference list

1. Ress-Loeschke, M., Fedrich, T., Hauer, B., Mattes, R., and Engels, D. (BASF AG), DE 19848129 [*Chem. Abstr.* **2000**, 132, 292813]. 2000.
2. Yamaguchi, Y., Ushigome, M., and Kato, T. (Nitto Chem. Ind.) EP 773297 [*Chem. Abstr.* **1997**, 127, 4190]. 1997.
3. Endo, T. and Tamura, K. (Nitto Chem. Ind.) EP 449648 [*Chem. Abstr.* **1992**, 116, 5338]. 1991.
4. Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, 348, 1655-1661.
5. Cao, L. Q.; van Rantwijk, F.; Sheldon, R. A. *Org. Lett.* **2000**, 2, 1361-1364.
6. Mateo, C.; Palomo, J. M.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Biotechnol. Bioeng.* **2004**, 86, 273-276.
7. van Langen, L. M.; Selassa, R. P.; van Rantwijk, F.; Sheldon, R. A. *Org. Lett.* **2004**, 7, 327-329.
8. Kiziak, C.; Conradt, D.; Stolz, A.; Mattes, R.; Klein, J. *Microbiology-Sgm* **2005**, 151, 3639-3648.
9. Rustler, S.; Müller, A.; Windeisen, V.; Chmura, A.; Fernandes, B. C. M.; Kiziak, C.; Stolz, A. *Enzyme Microb. Technol.* **2007**, 40, 598-606.
10. Sheldon, R. A. In *Biocatalysis in the Pharmaceutical and Biotechnology Industries*; R.N.Patel Ed. Immobilization of Enzymes as Cross-Linked Enzyme Aggregates: A Simple Method for Improving Performance. CRC Press: 20087; pp. 351-362.
11. Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2003**, 42, 3336-3337.
12. Mateo, C.; Fernandes, B.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *J. Mol. Catal. B: Enzym.* **2006**, 38, 154-157.
13. Gerrits, P. J.; Willeman, W. F.; Straathof, A. J. J.; Heijnen, J. J.; Brussee, J.; van der Gen, A. J. *Mol. Catal. B: Enzym.* **2001**, 15, 111-121.
14. Bradford, M. M. *Anal. Biochem.* **1976**, 72, 248-254.

**Cross-Linked Enzyme Aggregates
of Three Enzymes (triple-CLEA)**

4

1. Introduction

In the previous Chapter we described the co-immobilization of *M. esculenta* (S)-hydroxynitrile lyase (MeHnL) [E.C. 4.1.2.10] and nitrilase [E.C. 3.5.5.1] from *Pseudomonas fluorescens* EBC191 (PfNLase) into a combined - cross-linked enzyme aggregate (combi-CLEA). The immobilizate was used in the synthesis of enantiomerically pure (S)-mandelic acid (Figure 1).¹

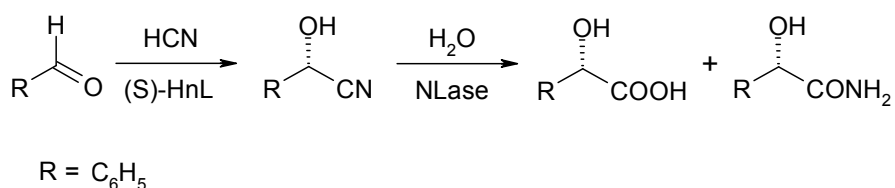


Figure 1. The bienzymatic procedure for the synthesis of (S)-mandelic acid, using an (S)-specific HnL and a nonspecific NLase in tandem.

In addition to (S)-mandelic acid, the product fraction also contained (S)-mandelic amide. Depending on the reaction conditions, the (S)-mandelic amide content could reach even 40% of the product. This nitrile hydratase (NHase) side activity was investigated as reported in Chapter 6, which described the correlation between the reaction conditions and the NLase – NHase switch.² The formation of the (S)-mandelic amide was shown to be catalyzed by the NLase and not by a NHase impurity in the NLase sample. One approach which would lead to the solution of this selectivity problem is to reach for the genetic toolbox. Using it, the NLase could be genetically modified to eliminate the NHase activity.

An alternative solution would be to use, in addition to the PfNLase, an additional enzyme with amide hydrolyzing properties. This latter protein will *in situ* hydrolyze the formed amide into the corresponding acid (Figure 2). A similar approach to overcome the problem with accumulating amide was already proposed and tested by Vejvoda *et al.*³

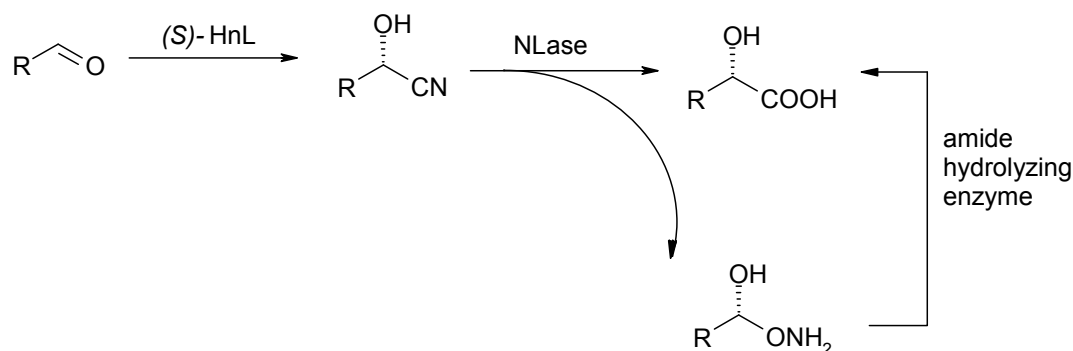


Figure 2. The pathway for the synthesis of enantiopure α -hydroxy acids using three enzymes.

In this Chapter we report the screening for a suitable amide hydrolyzing enzyme and the development of a method to co-immobilize the three selected enzymes as a triple-CLEA. A scheme for assaying the activity of the particular enzymes, as well as the application of the co-immobilizate in benzaldehyde conversions will be described. Finally, a protocol for increasing the storage stability of the triple-CLEA was developed.

2. Results and discussion

2.1. Screening for amide hydrolyzing enzymes

The screening for a suitable amide hydrolyzing enzyme which would fit in the universal platform for the synthesis of enantiomerically pure α -hydroxy acids (Figure 2), was carried out using a model reaction with (R,S) -mandelic amide as the substrate in citrate buffer pH 5.5 (Figure 3). Because of the planned application of the chosen enzyme in the multi-enzymatic process, the ideal amidase should be stable and active at pH 5.5 but not necessarily enantioselective.

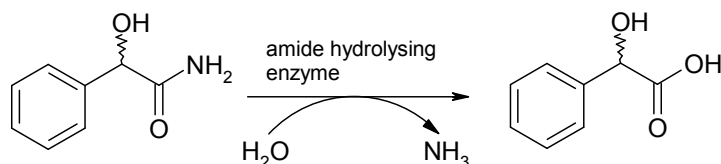


Figure 3. Amidase catalyzed hydrolysis of (R,S) -mandelonitrile.

A collection of enzymes from different origins (Table 1) which would be expected to have the desired properties, was studied. In the experiments (R,S) -mandelic amide

was incubated with catalytic amounts of the enzymes at 25 °C. The observed conversions and product enantiopurities are presented in Table 1.

Table 1. Activity of possible amide hydrolyzing enzymes in (*R,S*)-mandelic amide hydrolysis.

Enzyme name	ee [%(<i>R</i>)] acid (conv [%])
Penicillin acylase (<i>E. coli</i>), soluble ⁵	0 (100)
Penicillin V-acylase (Semacylase), (Novozymes)	-
Penicillin G acylase, immobilized ⁴	17 (75)
Penicillin G amidase, immobilized (Roche)	0 (100)
Penicillin acylase (<i>Alcaligenes f.</i>), immob. on silica	27 (45)
<i>Rhodococcus erythropolis</i> MP50 amidase ⁶	0 (100)

Reaction conditions as described in the experimental part

From the five enzymes which, in the above tests, converted (*R,S*)-mandelic amide into (*R,S*)-mandelic acid, two were selected and tested in a bienzymatic conversion of benzaldehyde in the presence of MeHnL and PfNLase combi-CLEA (as presented in Figure 2). Immobilized penicillin-G acylase and MP50 amidase were added into the biphasic reactions (90% diisopropyl ether (DIPE)) with the combi-CLEA. The reactions were monitored over time to see if (*S*)-mandelic amide formed in the course of the bienzymatic reaction was converted into the corresponding (*S*)-mandelic acid. The experimental data (not presented) showed that indeed, in both cases, after overnight reactions, all benzaldehyde and (*S*)-mandelic amide were converted into (*S*)-mandelic acid.

The final choice of enzyme for subsequent investigation was based on the substrate specificity and the availability of the protein. It was decided to use exclusively *Rhodococcus erythropolis* MP50 amidase. The enzyme has been isolated, cloned and expressed in JM109 *E.coli* by Stolz and his co-workers.⁶⁻⁸

2.2. MP50 amidase precipitation and stability studies of the aggregates

The initial experiments to aggregate and cross-link MP50 amidase under conditions that we commonly use (aggregation in 1,2-dimethoxyethane and cross-linking with dextran polyaldehyde)¹ failed. In these experiments all the activity in the cross-linked amidase was lost.

Hence, step by step studies to develop the triple-CLEA were carried out. First, in aggregation tests, a common suitable aggregation agent for all three enzymes was selected. In the following experiments the stability of the aggregates in the chosen precipitation agent were investigated.

2.2.1. MP50 amidase precipitation studies

Thirteen agents commonly used for enzyme precipitation were tested.⁹ Cell-free MP50 amidase samples were treated with aggregation agent, centrifuged and redissolved in phosphate buffer pH 7. The activity of the redissolved protein was measured (Table 2).

Table 2. Aggregation of MP50 amidase in different aggregation agents and the redissolved enzyme activity recovery.

Precipitation agent	Aggregation	Relative activity [%]	Activity in the supernatant
Free enzyme		100	
MeOH	+	0	0
EtOH	+	0	0
1-propanol	+	0	0
2-propanol	+	0	0
<i>tert</i> -butyl alcohol	+	45	0
acetone	+	0	0
acetonitrile	++	45	0
1,2-dimethoxyethane	+	40	0
ethyl lactate	+	0	0
sat. (NH ₄) ₂ SO ₄	+	49	0
dimethylformamide	+	0	0
dimethyl sulfoxide	-	n.d.	n.d.
polyethylene glycol (>50%)	-	n.d.	n.d.

Not determined (n.d.), precipitation (+), strong precipitation (++), no precipitation (-)
Reaction conditions as described in the experimental part

After redissolving the aggregated protein in phosphate buffer (we found it much more difficult to redissolve the aggregates in citrate buffer) and measuring the enzyme's

activity again, there was activity recovered only from tert-butyl alcohol, acetonitrile, 1,2-dimethoxyethane and sat. $(\text{NH}_4)_2\text{SO}_4$.

2.2.2. MP50 amidase aggregate stability

Next, the stability of the MP50 amidase aggregates in the precipitation agents was tested. A number of small aggregates (cell-free enzyme solution in 90% (v/v) tert-butyl alcohol, acetonitrile, 1,2-dimethoxyethane and sat. $(\text{NH}_4)_2\text{SO}_4$) were prepared and incubated on the shaker at 25 °C in several solvents. At different time intervals the activity upon the aggregate's redissolution was checked (Figure 4).

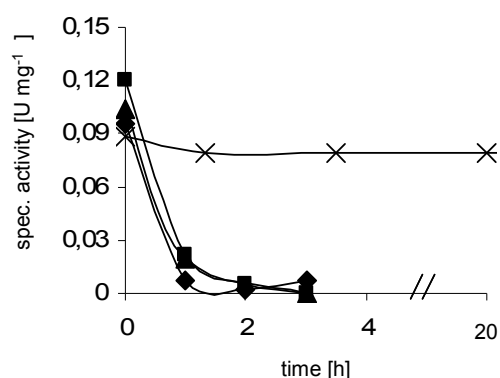


Figure 4. The activity of aggregated MP50 amidase in different aggregation agents over time. Tert-butanol (◆), 1,2-dimethoxyethane (▲), acetonitrile (■), sat. $(\text{NH}_4)_2\text{SO}_4$ (×).

Promising results were obtained in the aggregate stability experiment with sat. $(\text{NH}_4)_2\text{SO}_4$. Unlike experiments with tert-butyl alcohol, acetonitrile and 1,2-dimethoxyethane, the aggregate in sat. $(\text{NH}_4)_2\text{SO}_4$ maintained its activity during the incubation period. After 20 h the activity was still close to the initial value ($0.09 \text{ U} \cdot \text{mg}^{-1}$), whereas the other aggregates were less stable (after 1 h the values for acetonitrile and 1,2-dimethoxyethane dropped to $0.02 \text{ U} \cdot \text{mg}^{-1}$ and for tert-butyl alcohol it was only $0.01 \text{ U} \cdot \text{mg}^{-1}$). After 3 h of incubation in tert-butyl alcohol, acetonitrile and 1,2-dimethoxyethane, no or marginal activity was found. Having established that sat. $(\text{NH}_4)_2\text{SO}_4$ was suitable for MP50 amidase, it was investigated whether it also is suitable for aggregating MeHnL and PfNLase.

2.2.3. Stability of *M. esculenta* HnL and EBC191 NLase in saturated $(\text{NH}_4)_2\text{SO}_4$

The samples of cell-free MeHnL and PfNLase were aggregated in 90% (v/v) sat. $(\text{NH}_4)_2\text{SO}_4$. Figure 5 shows the stability of the redissolved enzymes from the incubation experiment with sat. $(\text{NH}_4)_2\text{SO}_4$.

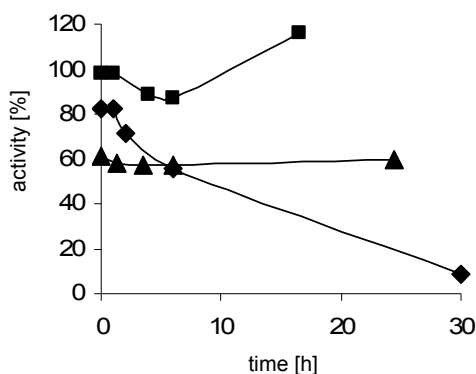


Figure 5. The stability of aggregated (S)-HnL, EBC191 NLase and MP50 amidase in sat. $(\text{NH}_4)_2\text{SO}_4$. EBC191 NLase (♦), MP50 amidase (▲), *M. esculenta* (S)-HnL (■).

This showed that sat. $(\text{NH}_4)_2\text{SO}_4$, was also an effective and safe aggregation agent for these two enzymes. There was high activity recovery (nearly 100%) observed from the redissolved MeHnL, as already reported previously.¹⁰ During the same treatment, MP50 amidase lost 40% and PfNLase 19% of its native activity. In the case of the NLase this was caused to a considerable degree by incomplete enzyme aggregation (data not shown). On the other hand, complete aggregation was observed in the HnL and amidase aggregation tests.

Little activity was lost over the first hour of incubation of the aggregates. Subsequently, the activity of the NLase was systematically dropping. MeHnL and MP50 amidase, in contrast, maintained their activity under the test conditions. Good stability of the aggregates is the first condition that must be fulfilled in the crosslinking procedure. The parameter is particularly important in cases when the cross-linking step (and thus stabilization) of the cross-linked protein is not immediate. Having a good aggregation agent in which the three enzyme aggregate activities are preserved, allowed us to start on the cross-linking step studies.

2.3. Cross-linking studies of single enzymes

In the tests, equal amounts of the three proteins were aggregated with sat. $(\text{NH}_4)_2\text{SO}_4$ at pH 8.7, which was chosen to bring the value closer to the physiological (optimum) pH of PfNLase and MP50 amidase. The aggregates were subsequently cross-linked with different volumes of dextran polyaldehyde solution. The cross-linking time varied from 2 h up to 23 h. The CLEAs and supernatant (activity leakage) were checked for activity and compared to the activity of the corresponding free enzyme.

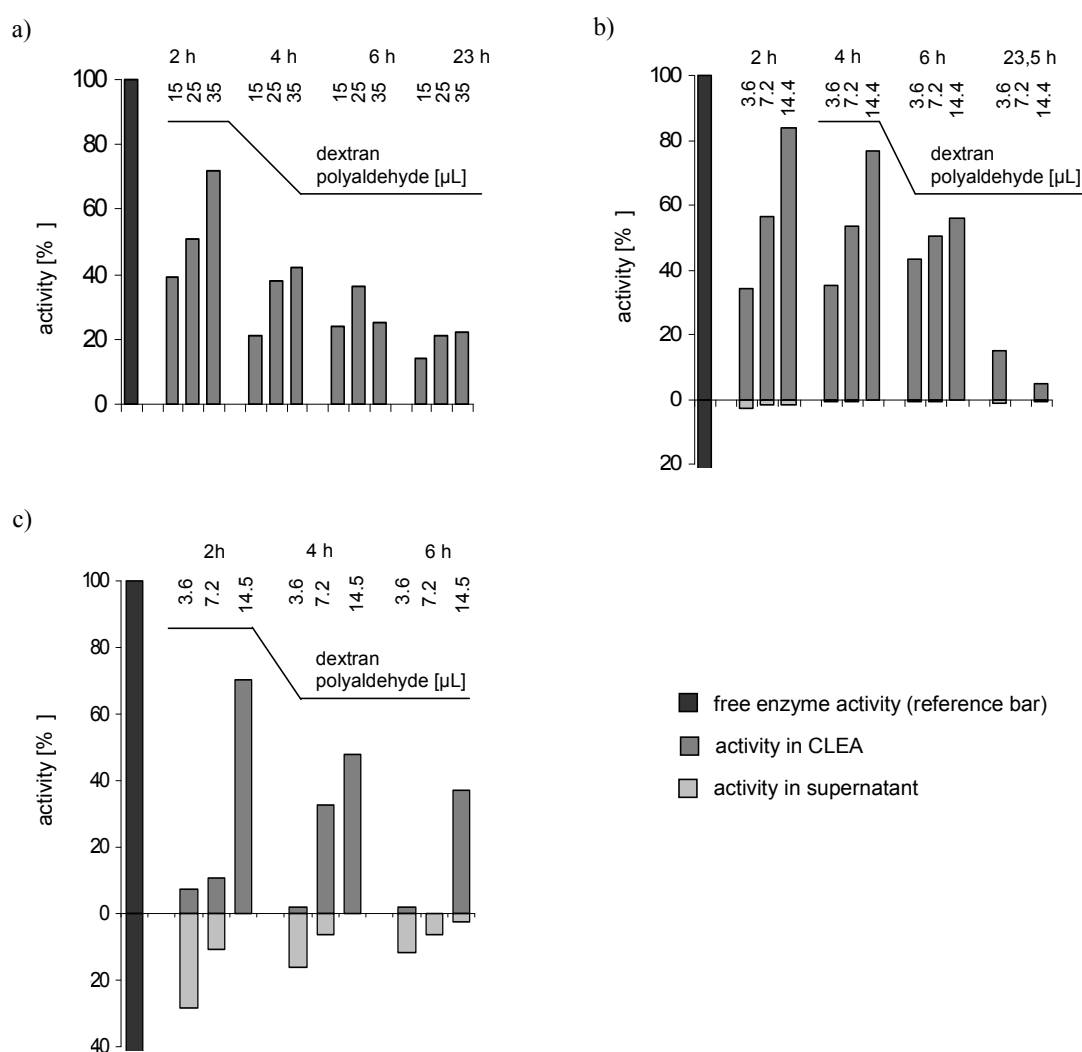


Figure 6. The influence of different amounts of dextran polyaldehyde and times of crosslinking on activity recovery from a) *M. esculenta* HnL CLEA, b) EBC191 NLase and c) MP50 amidase.

In all cases insoluble and catalytically active single-enzyme CLEAs were obtained. The highest activity recovery with the HnL CLEA (70%) (Figure 6a) was found after

2 h of crosslinking with 35 μL dextran polyaldehyde. A slow activity decay of the preparations was observed when cross-linking was continued for longer times. No leakage of activity was detected in the supernatant. The highest activity recovery in the NLase CLEA (85%) was found after 2 h of cross-linking with 14.4 μL dextran polyaldehyde (Figure 6b). Marginal amounts of active NLase were found in the supernatant. The amidase CLEA formation resulted in 70% activity recovery, compared with the activity of the free enzyme (Figure 6c). This was achieved, as with the other two enzymes, after 2 h with 14.5 μL dextran polyaldehyde. The activity decrease of the cell-free amidase extract from the supernatant after 4 and 6 h can be explained either by progressing (and therefore not immediate) cross-linking of the enzyme or the slow deactivation of the enzyme in the cross-linking media. In these test conditions, longer cross-linking times caused loss of activity in recovered CLEA, perhaps because cross-linking results in a complete loss of flexibility of the enzyme molecule, causing its deactivation.

2.4. Triple-CLEA preparation and the activity assay

2.4.1. Triple-CLEA preparation procedure

The averaged and semi-optimized conditions found in the previous experiments were used for preparing a triple-CLEA immobilizate according to the general procedure illustrated in Figure 7.

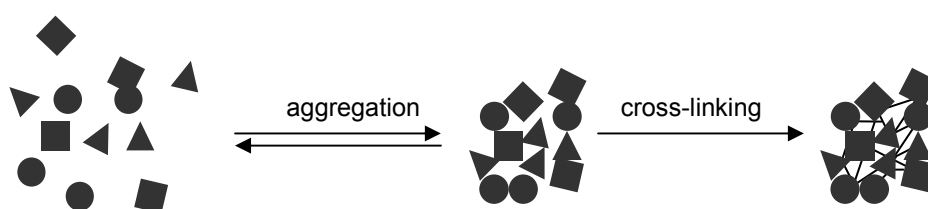


Figure 7. The general procedure for triple-CLEA preparation. *M. esculenta* HnL (■), EBC191 NLase (●), MP50 amidase (▲).

The first triple-CLEA batches were prepared by aggregating the enzymes sequentially in the following order: MeHnL, PfNLase and MP50 amidase, followed by cross-linking with dextran polyaldehyde. The fresh CLEA batch was reacted with NaCNBH_3 in order to reduce the Schiff's bases, washed and stored on ice as a

suspension in citrate buffer at pH 5.5. Later on, details in the protocol, such as the mixing order of the proteins were also investigated.

2.4.2. Activity assay of the triple-CLEA. Activity recovery in the triple-CLEA

The common activity assay methods of HnL, NLase and amidase are based either on spectrophotometric tests or on synthetic assays in which the assay results are determined by either GC or HPLC.^{8;10-12} Spectrophotometric tests are convenient, fast and therefore especially favored in situations where a high throughput is required.¹³ Spectrophotometric assays give excellent results in tests where free enzymes are used but, unfortunately, the methodology is not suitable when activities of solid enzyme preparations are measured due to too high signal noise caused by the presence of the solid particles. Additionally, spectrophotometric cuvette tests are not suitable in situations where two-phase assays are carried out.

The challenge in designing an activity assay for a multi catalyst preparation such as a triple-CLEA is finding proper conditions and substrates for the particular enzymes in the immobilizate. Especially, the careful selection of substrates is of special importance. Situations where a product of one activity measurement could become a substrate for the following catalyst must be avoided. The first assay, where HnL (enz. 1) activity is measured (Figure 8a), starts with the substrate A, giving product B. If the latter is in the substrate scope of the NLase (enz. 2), enz. 2 will immediately start utilizing B; consequently, if the rate of the latter reaction is appreciable, it will influence the reaction rate of enz. 1 (by shifting the equilibrium to the right). Analogously, when assaying the activity of enz. 2 (Figure 8b), the substrate C, the product D and when the NLase has double activity and converts C into both D+E, none of these compounds should be reactive with enz. 1 or amidase (enz. 3). The substrate choice for enz. 3 assay (Figure 8c) is relatively easy since the reactants for the enzyme – amides, do not react with enz. 1 or enz. 2 under the reaction conditions. Enz. 3 hydrolyses substrate F into G.

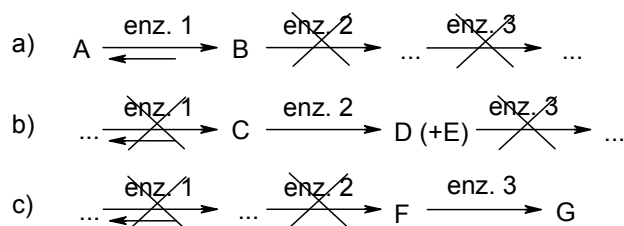


Figure 8. The general scheme for triple-CLEA activity assay.

For this trio, *trans*-cinnamaldehyde was found to be a good candidate as the substrate for MeHnL assay. The enzyme shows a good activity with this aldehyde, and even though PfNLase shows some activity with phenyl-2-hydroxy-3-butenenitrile (the hydrocyanation product), the reaction rate is minute compared with hydrocyanation and will have no influence on the HnL kinetics. Phenylacetonitrile would seem a good substrate for the NLase assay as it is not dehydrocyanated, and no phenylacetamide is formed under the hydrolysis conditions. Finally, (*R,S*)-mandelic amide can be used for measuring the activity of MP50 amidase. In the amidase catalyzed reaction, (*R,S*)-mandelic amide will be hydrolyzed to ammonium mandelate. The results of the enzyme assays are collected in Table 3.

Table 3. The recovered activity in triple-CLEA.

	Enz. spec. activity [U mg ⁻¹]		Activity recovery [%]
	Free enzyme	Triple-CLEA	Triple-CLEA
MeHnL	0.44	0.2	45
PfNLase	12.9	7.5	58
MP50 amidase	0.14	0.08	57

Reaction conditions as described in the experimental part

The cross-linking with dextran polyaldehyde resulted, as expected, in high enzyme activity recoveries. The recovered activity was 45% for MeHnL, 58% for PfNLase and 57% for the amidase. No activity was found in the crosslinking supernatant.

2.4.3. The application of a triple-CLEA in the synthesis of (S)-mandelic acid. (Comparison to combi-CLEA process)

The newly prepared triple-CLEA was tested in a reaction with 10 mM benzaldehyde in a biphasic system (50:50) with diisopropyl ether (DIPE) as the nonaqueous phase. For comparison an identical reaction was run with the combi-CLEA, with no amidase in the immobilizate (Figure 9). While converting benzaldehyde with the triple-CLEA, ideally no mandelic amide should be detected in any stage of the reaction. This can only be achieved when MP50 amidase activity is higher than the nitrile hydratase activity of PfNLase. The rational composition of the triple-CLEA was based on comparing the activities of the individual enzymes with particular enzymes with correction for the activity loss in the crosslinking procedure.

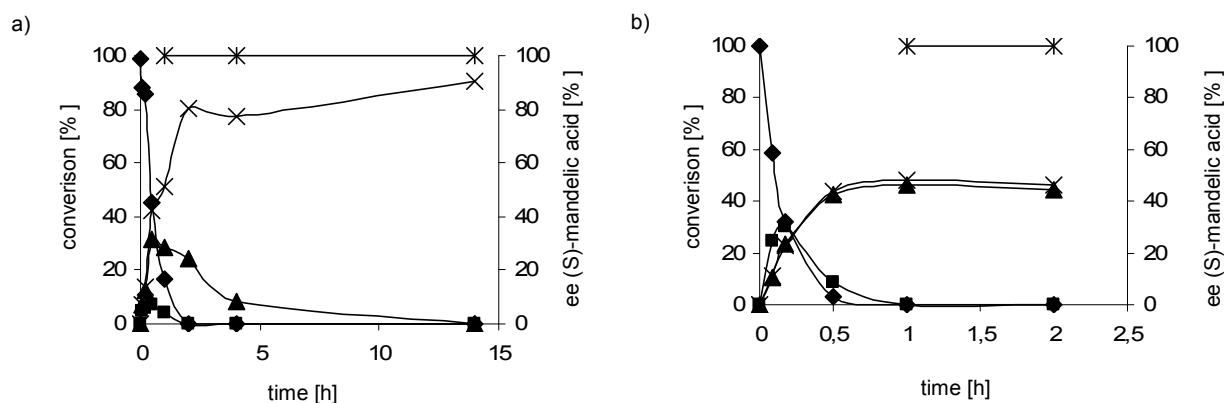


Figure 9. The comparison of a) triple-CLEA and b) combi-CLEA in the synthesis of enantiomerically pure (S)-mandelic acid from benzaldehyde. Benzaldehyde (◆), mandelonitrile (■), mandelic amide (▲), mandelic acid (×), (S)-mandelic acid ee (✱).

As shown in Figure 9a, benzaldehyde was rapidly converted into mandelonitrile, which was removed at nearly equal rate – very little nitrile accumulated – by the NLase. Some mandelic amide accumulated and required 15 h to be completely converted. Eventually benzaldehyde was indeed fully converted into (S)-mandelic acid in the presence of the triple-CLEA. The enantiomeric purity of the (S)-mandelic acid was >99%. The same reaction in the presence of the combi-CLEA (Figure 9b), resulted in nearly equal amounts of (S)-mandelic acid and (S)-mandelic amide, as discussed in Chapter 3.

2.5. Triple-CLEA stability. Different approaches in the immobilizate preparation

2.5.1. Storage stability. Storage under Ar

All NLases possess several conserved sequence motifs and invariably contain a cysteine residue in the catalytic center.¹⁴⁻¹⁶ NLases are well known to be sensitive to oxygen, which is commonly ascribed to the autoxidation of these cysteine residues. Therefore, in principle, it should be possible to avoid the oxygen inactivation of a NLase by storing the enzyme in an oxygen-free atmosphere.¹⁴ In these experiments the stability of the triple-CLEA was compared on storing the suspended immobilizate under air or an inert gas. For the experiment, a batch of freshly prepared triple-CLEA was divided into two portions and in one of these, oxygen was substituted by Ar. After several days of storage, the ice-stored triple-CLEAs were assayed for their activities. The values were compared to the enzyme activities in the fresh immobilizate (Table 4).

Table 4. Stability of the triple-CLEA in oxygen-free atmosphere.

	Specific activity [U mg ⁻¹]		
	Fresh	6 days	
		Air	Ar
MeHnL	0.1	0.1	0.1
PfNLase	7.8	3.7	3.5
amidase	0.05	0.04	0.04

Reaction conditions as described in the experimental part

The experiment revealed that the deactivation of PfNLase in the triple-CLEA is not related to the presence of oxygen. The activity of the NLase in both batches, after six days, dropped to < 50% of its initial activity (7.8 U·mg⁻¹). Because of the latter, it was concluded that in the triple-CLEA the NLase activity loss is not caused by the inactivating effect of oxygen. This indicates that another mechanism than cysteine oxidation must be involved in the coimmobilized PfNLase deactivation process. MeHnL and amidase on the other hand, retained they initial activities.

2.5.2. Storage stability in different solvents

Using citrate buffer pH 5.5 as the storage solvent is of a practical value because the buffer commonly used as the reaction medium in the triple-CLEA experiments. On the other hand, using a solvent such as saturated aqueous $(\text{NH}_4)_2\text{SO}_4$ which is a known enzyme stabilizer, for storing the coimmobilizate, could possibly improve the storage properties of the triple-CLEA. In these experiments the fresh triple-CLEA was divided into two equal portions. One portion was resuspended in citrate buffer pH 5.5, and the other in sat. $(\text{NH}_4)_2\text{SO}_4$ at pH 8.7. The samples were stored on ice. Activities of the particular enzymes were assayed occasionally (Figure 10).

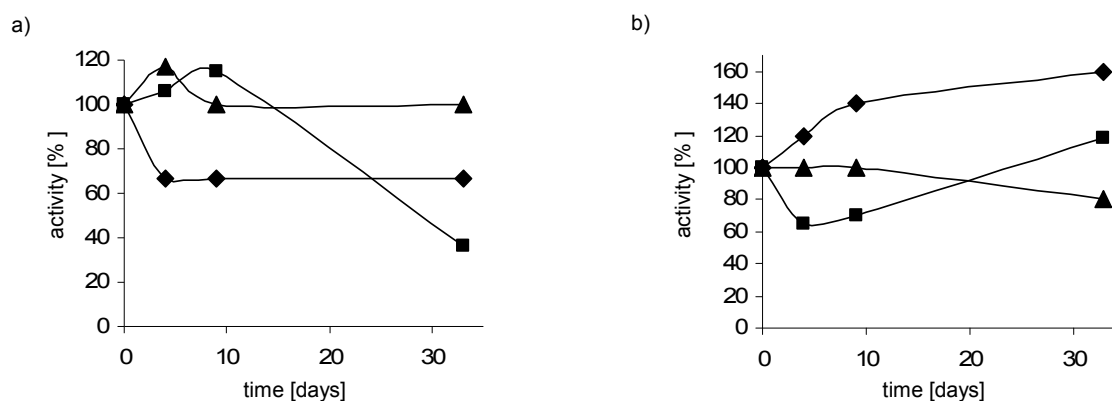


Figure 10. The triple-CLEA stability in a) citrate buffer and b) saturated $(\text{NH}_4)_2\text{SO}_4$. MeHnL (◆), PfnLase (■), amidase (▲).

Storage of the triple-CLEA in citrate buffer at pH 5.5 resulted in a significant change of PfnLase activity. During the first 9 days, the enzyme gained 15% activity, but after that it started to drop, losing 66% its initial activity after 33 days. The storage also effected MeHnL and 33% of its initial activity was lost in the first four days. The value however, remained unchanged afterwards. The co-immobilized amidase was stable in citrate buffer during the whole storage period. A significantly different behavior of the triple-CLEA regarding its activity was observed with the batch stored in sat. $(\text{NH}_4)_2\text{SO}_4$ at pH 8.7. The activity of PfnLase, after its initial drop, reached 118%, compared to its initial activity, after 33 days of the storage. A 20% activity drop after the same storage period was observed in the case of the amidase. The activity of MeHnL was constantly increasing, reaching 160% of its initial activity after 33 days. The storage experiments showed that a medium such as sat. $(\text{NH}_4)_2\text{SO}_4$ is strongly

preferred for storing the triple-CLEA over citrate buffer, probably due to the difference in pH combined with the stabilizing effect of sat. $(\text{NH}_4)_2\text{SO}_4$.

2.5.3. Crosslinking with different enzyme aggregation orders

In the course of the immobilization procedure the enzymes are exposed to ammonium sulfate and dextran polyaldehyde, possibly leading to partial deactivation. Hence, we surmised that aggregating the sensitive enzymes first, followed by the more stable enzymes, could help to protect the more fragile proteins. Would it be possible to obtain a protecting, coating effect around the sensitive enzymes?

The concept to recover more activity in triple-CLEA was investigated by either premixing the enzymes, and precipitating them as the mixture (2) or, aggregating the enzymes by adding first the most sensitive enzymes and ending with MeHnL as the most stable one, prior to cross-linking (3). This was compared to the “traditional” process in which first MeHnL is added, followed by co-aggregation of PfNLase and amidase (1) (Table 5).

Table 5. Comparison of triple-CLEAs with variable aggregation order.

	Specific activity [U mg^{-1}]			
	Free enzyme	1	2	3
MeHnL	0.18	0.10	0.09	0.072
PfNLase	13.0	6.05	6.11	9.10
MP50 amidase	0.10	0.033	0.032	0.033

Mixing order: HnL, NLase, amidase (1), enzymes premixed prior to aggregation (2), amidase, NLase, HnL (3).

Premixing the enzymes (2) does not bring any change in activities recovered in triple-CLEA, compared to the standard procedure (1). Reversing the aggregation order (method 3) however, gave a significantly increased (25% improved) activity recovery of PfNLase and a somewhat lower activity recovery of MeHnL. The activity recovery of the amidase remained at the same level.

2.5.4. Triple-CLEA drying in organic solvents

Theoretically, stabilization of the co-immobilizate could also be achieved by separating the CLEA from the storage solvent by e.g. lyophilizing (not tested) or drying it with volatile, organic solvents. In this experiment, the citrate buffer was removed from the triple-CLEA samples by washing with either ethanol or acetone. The buffer-free CLEA samples were left to dry. The dry samples were again resuspended in citrate buffer pH 5.5 and assayed for activity (Table 6).

Table 6. The activity recoveries from triple-CLEA drying with ethanol and acetone.

	Specific activity [U mg ⁻¹]		
	Original	Ethanol	Acetone
MeHnL	0.11	0.09	0.10
PfNLase	11.7	1.1	5.1
MP50 amidase	0.05	0	0

Test conditions as described in the experimental part

Unfortunately, both solvents appeared to be highly toxic for the co-immobilized amidase as all the enzyme activity was lost during the washing/drying procedures. Acetone also showed a negative effect on PfNLase which cost the NLase 56% of its initial activity. MeHnL in contrast, was much more stable and its activity was hardly affected by the solvent treatment.

3. Conclusions

We introduced the concept of a triple-CLEA as a solution for the formation of the amide as a byproduct of the double activity of the NLase in the synthesis of (S)- α -hydroxy acids. We screened for suitable amide hydrolyzing enzymes, finding in a small library of six enzymes, MP50 amidase to be most suitable from those tested. The enzymes were successfully co-immobilized in the form of a triple-CLEA. A method to assay the activities of the particular enzymes in the three-enzyme immobilizate was developed.

The rationally designed triple-CLEA was successfully used in the synthesis of (S)-mandelic acid. In the reaction, the co-immobilized MP50 amidase simultaneously

hydrolysed the formed (S)-mandelic amide to the corresponding hydroxy acid. A major issue was the triple-CLEA storage stability while stored in citrate buffer. A significant increase in stability of the triple-CLEA was achieved by storing the coimmobilizate in sat. $(\text{NH}_4)_2\text{SO}_4$ pH 8.7. Moreover, we found that more activity can be recovered in triple-CLEA, aggregating the enzymes one by one, starting with the most sensitive ones. In the result, a coating effect is observed.

4. Materials and methods

Chemicals

Semi-purified (S)-hydroxynitrile lyase from *M. esculenta* [E.C. 4.1.2.10] (protein content 88 mg mL⁻¹) was obtained from Jülich Fine Chemicals (Jülich, Germany). *E. coli* JM109 heterologously expressed EBC191 NLase from *Pseudomonas fluorescens* [E.C. 3.5.5.1] was obtained as described.¹⁷ The NLase cell-free extract contained 34 mg protein mL⁻¹. MP50 amidase from *Rhodococcus erythropolis* [E.C. 3.5.1.4] was heterologously produced in *E. coli* JM109.⁶ Protein content was 19.5 mg protein·mL⁻¹. Phenylacetonitrile and phenylacetic acid were obtained from MERCK-Schuchardt, (R,S)-mandelic amide 97% from Alfa Aesar. (R,S)-Mandelic acid and *trans*-cinnamaldehyde +99% from Acros Organics. Veratrole +99% and DIPE were purchased from Fluka. The aggregation agents were from commercial sources (Acros, Janssen Chemica or Sigma-Aldrich). All the chemicals were used as provided by suppliers, without further purification. Dextran polyaldehyde: was obtained by oxidation of Dextran 100-200 kDa (Serva Feinbiochemica) as described.¹⁸ HCN was prepared as previously described.¹⁰ 2-hydroxy-4-phenyl-*trans*-3-butenitrile (modified procedure¹⁹): 0.02 mol of freshly distilled *trans*-cinnamaldehyde was reacted overnight in 0.1 M phosphate buffer pH 7.5 with 0.6 mol HCN (2 M solution in DIPE) (added dropwise) in a magnetically stirred reactor. When the reaction reached equilibrium (> 90% conversion), the cyanohydrin was extracted into CH₂Cl₂/hexane (50:50) and dried over MgSO₄. Solvent was removed in vacuo until crystallization occurred spontaneously. The crystals were filtered off and dried. Fine, yellowish crystals were obtained. The configuration of the product was confirmed by ¹H NMR.

Instrumentation

The reaction progress of all of the reactions was followed by HPLC - Alliance Waters 2695 Separation Module, equipped with Waters 2487 Dual λ Absorbance Detector. The separations were achieved with 4.6 x 50 mm Merck ChromolithTM SpeedROD RP-18e column and H₂O:ACN (90:10) + 0.1% (v/v) TFA as eluent (1 mL·min⁻¹). The detector was set at 215 nm. The enantiomeric purity of (*R,S*)-mandelonitrile and the corresponding hydrolysis products were measured on HPLC system equipped with Waters 515 pump and Waters 486 Tunable Absorbance Detector, set at 215 nm. The separation was achieved with the straight phase 4.6 x 250 mm Chiralpak AD-H column and hexane:isopropanol (80:20) + 0.1% (v/v) TFA as eluent.

Kinetic assay experiments were carried out on Q.Instruments ThermoTWISTER comfort shaker.

Protein content measurements

The total protein concentration of cell-free extracts were assayed according to the standard Bradford procedure.²⁰ The samples were measured on Shimadzu UV-240IPC spectrophotometer.

Screening for amide hydrolyzing enzyme

Catalytic amounts of the enzymes were diluted or suspended in 0.5 mL 0.1 M (*R,S*)-mandelic amide solution (10 mM) in 20 mM citrate buffer pH 5.5, with 0.5 mL DIPE as the second phase. Samples for conversion measurements were taken occasionally from the aqueous phase and analyzed by HPLC. The samples for enantiomeric purity measurements, were first extracted from the aqueous phase to ethyl acetate and then measured on the straight phase column.

The initial conversions with three enzymes: 500 μ L combi-CLEA with small portions of immobilized penicillin G-amidase and in a separate experiment with MP50 amidase, were mixed with 3.6 mL DIPE in 10 mL magnetically stirred glass reactor. HCN (50 mM), IS (benzoic acid) (10 mM) and benzaldehyde (10 mM) were added to start the reaction. The reaction temp. was 25 °C. Samples were analyzed as described in the previous paragraph.

Free enzyme kinetic assays

One unit (U) of an enzyme will convert 1 μ mol of a substrate per min at 25 °C.

Oxynitrilase: Into 1.5 mL eppendorf filled with 0.5 mL 20 mM citrate buffer pH 5.5, 5 μ L (0.44 mg protein) enzyme solution, 395 μ L DIPE, IS (veratrole) and HCN solution (0.2 M) were added. Finally, *trans*-cinnamaldehyde solution (10 mM) was added. The reaction was incubated on the shaker at 25 °C. The reaction vessel was kept closed to prevent the escape of HCN. 10 μ L samples were taken from each phase and analyzed on reversed-phase HPLC.

Nitrilase: 1.5 mL eppendorf was filled with 980 μ L 20 mM citrate buffer pH 5.5, 10 μ L (0.003 mg protein) 100x prediluted nitrilase solution, IS (veratrole) and finally phenylacetonitrile (10 mM) was added. 10 μ L samples were taken and analyzed on reversed-phase HPLC.

Amidase: 1.5 mL eppendorf was filled with 885 μ L 20 mM citrate buffer pH 5.5, 10 μ L (0.195 mg protein) enzyme solution, IS (veratrole) and finally (*R,S*)-mandelic amide (10 mM) was added. 10 μ L samples were taken and analyzed on reversed-phase HPLC.

Aggregation studies

The enzyme samples: 20 μ L (0.88 mg protein) MeHnL, 7.5 μ L (0.23 mg protein) PfNLase and 7.5 μ L (0.44 mg protein) MP50 amidase, were separately aggregated in 90% (v/v) aggregation agent (in 0.5 mL eppendorf tubes). After incubation for the times specified in the Figure 4 the aggregate was spun down at 11k rpm for 2 min and was redissolved in 20 mM citrate buffer pH 5.5 or 0.1 M phosphate buffer pH 7.0. The activities of the redissolved proteins and of the activity in the supernatants were assayed as described in the preceding section.

Single enzyme CLEA

MeHnL: 15 μ L (1.34 mg protein) enzyme samples were aggregated in 135 μ L cold, saturated $(\text{NH}_4)_2\text{SO}_4$ pH 8.7 in 1.5 mL eppendorfs. The aggregates were cross-linked by addition of different amounts of dextran polyaldehyde solution (3.2%) (15, 25 and 35 μ L). Cross-linking was carried out on the thermo-shaker set at 25 °C for 2, 4, 6 and 23 h. After the specified time, 5.2 mg NaCNBH_3 was added (dissolved in minimal amount of H_2O). The reaction was shaken for another 60 min. The final step, washing, was carried out by three resuspending-centrifugation cycles (13k rpm, RT) in 150 μ L H_2O . The activity assay of the resulting CLEA and the check for the activity in the cross-linking media was carried out directly after the washing step. The activity

assay was identical to that described in “the free enzyme kinetic assays” above, with the difference that here, benzaldehyde was used as the substrate. 10 μ L samples were taken from each phase and treated as described in the preceding section.

PfNLase: the immobilizate was prepared in an identical way as for MeHnL CLEA. The differences were in the amounts of used chemicals, and they were: 15 μ L enzyme (0.45 mg protein), dextran polyaldehyde volumes added: 3.6, 7.2 and 14.4 μ L, NaCNBH₃ added: 1.5 mg. The activity assay and the sample measurements were as described above.

Amidase: the immobilizate was prepared in an identical way as for MeHnL CLEA. The differences were in the amounts of used chemicals, and they were: 15 μ L enzyme (0.88 mg protein), dextran polyaldehyde volumes added: 3.6, 7.2 and 14.4 μ L, NaCNBH₃ added: 1.5 mg. The activity assay and the sample measurements were as described above.

Immobilization, semi-optimized triple-CLEA

0.25 mL (22 mg protein) MeHnL, 0.5 mL (17 mg protein) PfNLase and 2.5 mL (49 mg protein) amidase were aggregated in 24.5 mL cold, saturated (NH₄)₂SO₄ pH 8.7. The aggregates were cross-linked with 2.2 mL dextran polyaldehyde solution (3.2%) followed by magnetic stirring for 2 h at room temperature. Next, to the fresh triple-CLEA suspension, 412 mg NaCNBH₃ was added (dissolved in minimal amount of H₂O) to reduce the Schiff's bases. The reaction was shaken for another 60 min. Washing was carried out by triple resuspending-centrifugation cycles in 25 mL 20 mM citrate buffer pH 5.5 at 4k rpm at 4 °C. The triple-CLEA was stored on ice as a suspension in the citrate buffer.

Triple CLEA activity assay tests

The experiments were carried out in an identical way as those for the free enzymes. The amounts of a particular protein in mg, were adjusted such as to give the same amount of protein in the reaction vessel as those in the free enzyme assays.

Synthesis with the triple-CLEA

Triple-CLEA conversions: 30 μ L freshly prepared triple-CLEA, containing 0.03 U MeHnL, 1.7 U PfNLase and 0.03 U amidase, was mixed with 0.47 mL 20 mM citrate buffer pH 5.5 in a 1.5 mL eppendorf. To the enzyme suspension, 390 μ L DIPE, HCN

(0.2 M), veratrole (10 mM) and finally benzaldehyde (10 mM) were added to start the reaction. 10 μ L samples for conversion measurements were taken occasionally from both phases and analyzed by HPLC (method described in the instrumentation section). The samples for enantiomeric purity measurements were first extracted from the aqueous phase to ethyl acetate and then measured on the straight phase column (method described in the instrumentation section).

Reference reaction with combi-CLEA: the experiment was identical to the above one, but with the different numbers of units of particular enzymes: 0.09 U MeHnL and 3.2 U PfNLase.

Stability test

The preparation of the triple-CLEA (66 mg MeHnL, 51 mg PfNLase and 92 mg amidase) was performed as described above. After the Schiff's bases reduction step, the samples were divided into two equal portions and spun down to separate the immobilizate from the cross-linking media.

Stability under Ar: The immobilizate pellet was washed three times with 50 mL 20 mM citrate buffer pH 5.5. Additionally, from one portion oxygen was removed by bubbling the suspension with Ar for 15 min. The resuspended triple-CLEA samples were stored on ice. The activities of the particular enzymes were assayed according to the standard procedure.

Stability in different solvents: The immobilizate pellet was washed three times with 50 mL 20 mM citrate buffer pH 5.5, or with sat. $(\text{NH}_4)_2\text{SO}_4$ pH 8.7. The resuspended triple-CLEA samples were stored on ice. The activities of the particular enzymes were assayed according to the standard procedures.

Drying in organic solvents.

0.5 mL the triple-CLEA samples (prepared as described above), suspended in 20 mM citrate buffer pH 5.5, were transferred into 1.5 mL eppendorfs and spun down at 10k rpm for 0.5 min at 4 °C. The pellets were washed three times with 0.8 mL portions of acetone and in a separate experiment with EtOH. After the third washing, the triple-CLEA samples were resuspended in the organic solvents (0.5 mL) once more and left in a fume-hood for drying. For the activity assay, the dry triple-CLEA samples were resuspended in citrate buffer pH 5.5. The activities of the particular enzymes were assayed according to the standard procedures.

5. Reference list

1. Mateo, C.; Chmura, A.; Rustler, S.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Tetrahedron: Asymmetry* **2006**, *17*, 320-323.
2. Fernandes, B. C. M.; Mateo, C.; Kiziak, C.; Chmura, A.; Wacker, J.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 2597-2603.
3. Vejvoda, V.; Kaplan, O.; Kubáč, D.; Křen, V.; Martinková, L. *Biocatal. Biotransform.* **2006**, *24*, 414-418.
4. Ismail, H.; Madeira Lau, R.; van Langen, L. M.; van Rantwijk, F.; Švedas, V. K.; Sheldon, R. A. *Green Chem.* **2008**, *10*, 415-418.
5. Guranda, D. T.; Volovik, T. S.; Švedas, V. K. *Biochemistry-Moscow* **2004**, *69*, 1386-1390.
6. Trott, S.; Burger, S.; Calaminus, C.; Stolz, A. *Appl. Environ. Microbiol.* **2002**, *68*, 3279-3286.
7. Hirrlinger, B.; Stolz, A.; Knackmuss, H. J. *J. Bacteriol.* **1996**, *178*, 3501-3507.
8. Hirrlinger, B.; Stolz, A. *Appl. Environ. Microbiol.* **1997**, *63*, 3390-3393.
9. Schoevaart, R.; Wolbers, M. W.; Golubovic, M.; Ottens, M.; Kieboom, A. P. G.; van Rantwijk, F.; van der Wielen, L. A. M.; Sheldon, R. A. *Biotechnol. Bioeng.* **2004**, *87*, 754-762.
10. Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1655-1661.
11. Heinemann, U.; Engels, D.; Burger, S.; Kiziak, C.; Mattes, R.; Stolz, A. *Appl. Environ. Microbiol.* **2003**, *69*, 4359-4366.
12. Cabirol, F. L.; Hanefeld, U.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1645-1654.
13. Reymond, J. L. *Food Technol. Biotechnol.* **2004**, *42*, 265-269.
14. Mateo, C.; Fernandes, B.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *J. Mol. Catal. B: Enzym.* **2006**, *38*, 154-157.
15. Kobayashi, M.; Komeda, H.; Yanaka, N.; Nagasawa, T.; Yamada, H. *J. Biol. Chem.* **1992**, *267*, 20746-20751.
16. Bork, P.; Koonin, E. V. *Protein Sci.* **1994**, *3*, 1344-1346.
17. Kiziak, C.; Conradt, D.; Stolz, A.; Mattes, R.; Klein, J. *Microbiology-Sgm* **2005**, *151*, 3639-3648.
18. Mateo, C.; Palomo, J. M.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Biotechnol. Bioeng.* **2004**, *86*, 273-276.
19. Gerrits, P. J.; Willeman, W. F.; Straathof, A. J. J.; Heijnen, J. J.; Brussee, J.; van der Gen, A. J. *Mol. Catal. B: Enzym.* **2001**, *15*, 111-121.
20. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.

**A Novel, One Cell, Two Enzyme Biocatalyst.
The Biocatalytic Tests of Recombinant
E. coli which Simultaneously Express
an (S)-Oxynitrilase and a Nitrilase**

5

A part of the Chapter contents have been published in:

Sosedov, O.; Matzer, K.; Burger, S.; Kiziak, C.; Baum, S.; Altenbuchner, J.; Chmura, A.; Rantwijk, F. v.; Stolz, A. *Adv. Synth. Catal.* **2009**, 351, 1531-1538.

1. Introduction

Whereas genetic manipulation and bacterial overexpression of genes, such as those coding for enzymes, is a well established tool in microbiology (a first work of this kind was published in 1973 by Cohen *et. al.*¹), engineering a cell that will accommodate two enzymes is a novel approach and can be considered as a step forward in whole cell biocatalysis.

In this Chapter we present a two-enzyme, one-cell biocatalyst, constructed by simultaneously expressed genes encoding the plant derived *M. esculenta* oxynitrilase (MeHnL) and the bacterial *P. fluorescens* nitrilase (PfNLase) in a recombinant *E. coli* JM 109 strain² (double clone, see Figure 1b). A similar construct, expressing the same enzymes but in a recombinant derivative of the methylotrophic yeast *Pichia pastoris*, was already published by Rustler *et al.*³

In Chapters 3 and 4 we presented our efforts to obtain stable co-immobilized enzymes in the form of a combi- or triple-CLEA, and apply these in a one-pot, two-step conversion of benzaldehyde (**1**) into enantiomerically pure (*S*)-mandelic acid (**2**) with (*S*)-mandelic amide (**3**) as the by-product (see Figure 2). As found there, the enzyme environment such as pH and the nature of the organic solvent used as a second phase – to suppress the chemical hydrocyanation reaction and act as an HCN reservoir – play a crucial role in the final product purity as well as influencing the enzyme activities. Generally, better results were obtained when co-immobilized enzymes were used (Figure 1a) instead of separate immobilizates.

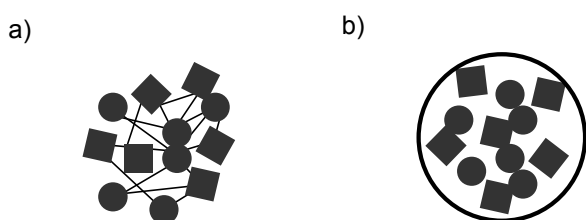


Figure 1. Schematic picture of two different multi-catalyst systems containing the same enzymes. Combi-CLEA (a), bacterial cell (b). HnL (■), NLase (●).

In this Chapter we will describe the application of the double clone biocatalyst in the one-pot synthesis of (*S*)-**2** set out above which represents an interesting alternative to synthesis with combi-CLEA composed of two or three enzymes described in the

previous Chapters. The effects of the reactant concentration and the medium (aqueous monophasic or aqueous-organic bi-phasic) will be investigated and its merits in practical synthesis will be discussed.

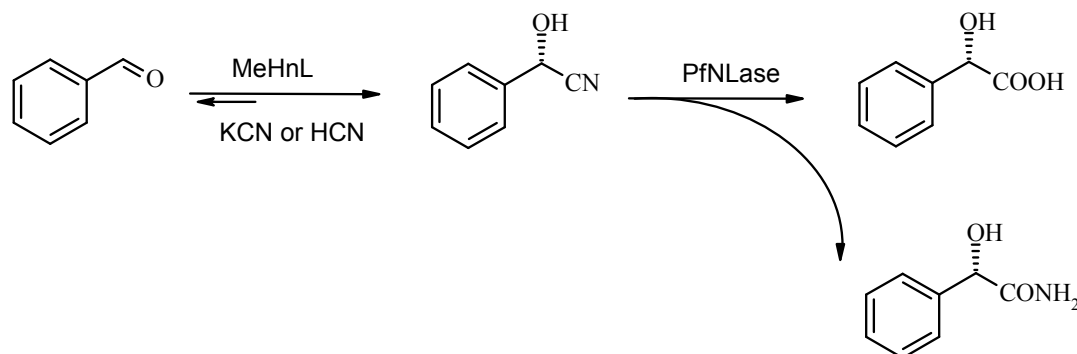


Figure 2. Two-step, one pot synthesis of (S)-2 catalyzed by one-cell MeHnL - PfNLase double clone.

2. Results and discussion

Mono-phasic conversions with whole cell biocatalysts in an aqueous buffer as the reaction medium represent a classical approach in biocatalysis. Such a natural enzyme environment allows precise pH control, for example, but the solubility of reactants is often poor. In this particular case, moreover, the spontaneous, undesired hydrocyanation of aldehydes or ketones, which is relatively fast in aqueous medium, has to be taken into account. A water-miscible co-solvent would improve the reactant solubility but have little effect on the background hydrocyanation.

Bi-phasic systems, on the other hand, could increase the over-all reactant solubility even more. The second, organic phase acts as a storage phase, separating the reactants (possible inhibitors) from the enzyme that remains in the aqueous phase. Furthermore, as discussed in Chapter 2, the uncatalyzed hydrocyanation is suppressed.

Water-miscible organic solvents as well as bi-phasic aqueous-organic systems can potentially have a toxic effect on the *E. coli* cells. The cell wall of gram-negative bacteria such as *E. coli* is made up of an outer membrane and a peptidoglycan layer that surrounds the cytoplasmic membrane. The outer membrane contains several major structural proteins, lipoproteins as well as some minor proteins and

lipopolysaccharides. Organic solvents may dissolve in the cell membrane, disturbing its integrity and effecting specific permeabilization. As a general rule, organic solvents with $\log P < 4.5$, such as diisopropyl ether (DIPE, $\log P$ 1.9) exhibit a toxic influence on cells.⁴⁻⁶

2.1. Conversions in monophasic medium

In the following experiments cells of the double clone were tested in 250 mM aqueous citrate buffer pH 5.0. The cells converted **1** (10 – 200 mM) and KCN into the corresponding (S)-**2** and (S)-**3** (Figure 3).

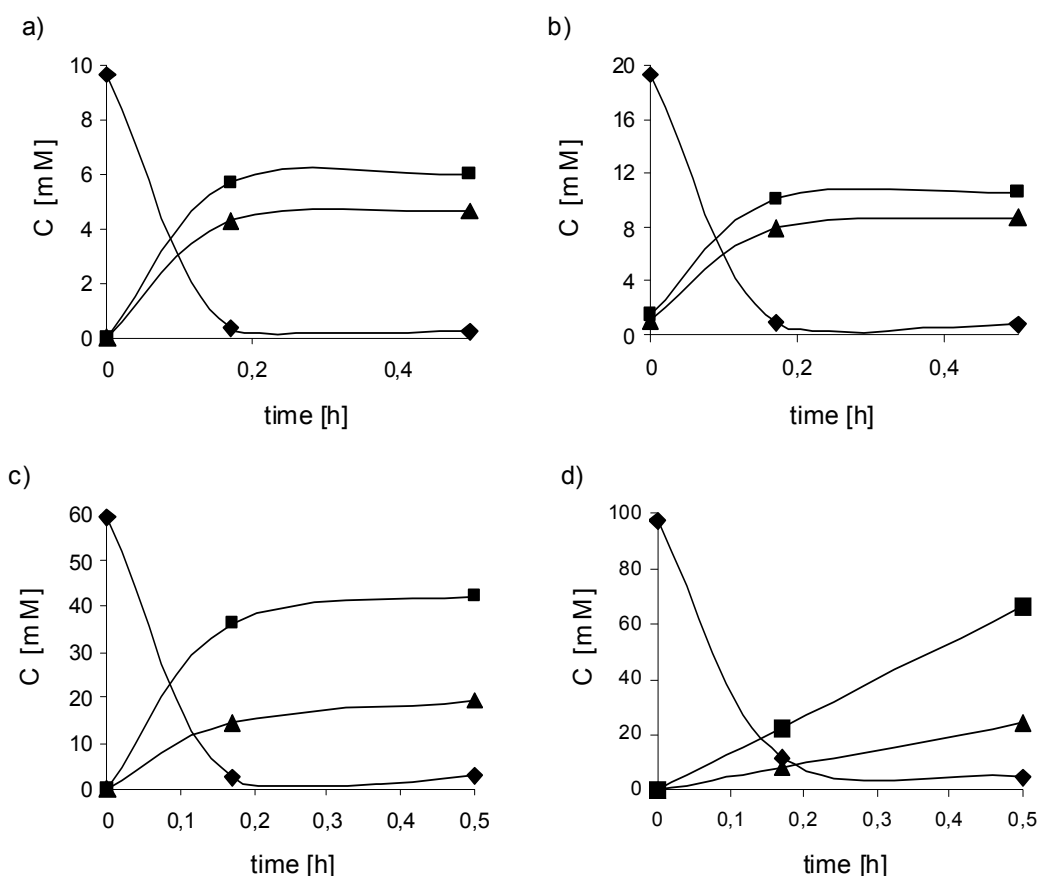


Figure 3. One-cell, *E. coli* expressed (S)-HnL and PfNLase in conversions of **1** (10 – 100 mM) and KCN (30 – 600 mM), a) – d) respectively, into (S)-**2** in mono-phasic reaction in 250 mM citrate buffer pH 5.0 and 25 °C. **1** (◆), **2** (■), **3** (▲).

Reactions with 10 – 50 mM **1** (Figure 3a – c) reached completion already in less than 0.2 h. With 0.1 M **1** (Figure 3d), in contrast, hydrocyanation was complete within 0.3 h, but nitrile hydrolysis lagged and still was incomplete at 0.5 h.

It should be noted here that KCN was used as the cyanide donor in the hydrocyanation step, which rendered the reaction medium alkaline because cyanide anion (pKa 9.31) is a moderately strong base (see Table 1).

Table 1. The change of the reaction medium pH after addition of KCN in the one-pot, two-step synthesis of (S)-**2** using one-cell *E. coli* expressed (S)-HnL and PfNLase in mono-phasic system.

1 [mM]	KCN [mM]	pH
10	30	5.5-6.0
20	60	5.5-6.0
50	150	9
100	300	11
200	600	13

25 °C, 250 mM citrate buffer pH 5.0

The pH shift to alkaline had a strongly negative influence on the enantiopurity of the final product due to the unselective uncatalyzed hydrocyanation, which competes with the desired enzymatic process, decreasing the enantiopurity of (S)-**2**, at pH > 6. Thus, at an initial concentration of **1** of ≤ 20 mM, (S)-**2** and (S)-**3** with ee > 99% were obtained. In reactions with > 20 mM **1**, in contrast, the ee(S) was < 50% (data not shown), which we ascribe to fast background hydrocyanation. At pH 13, furthermore, cell lysis occurs, destroying the enzymes. A separate experiment with more concentrated buffer (0.5 M) did not improve the results (data not shown).

Table 2. The effect of KCN concentration on acid/amide ratio in the one-pot, two-step synthesis of (S)-**2** using one-cell *E. coli* expressed (S)-HnL and PfNLase in mono-phasic system.

1 [mM]	KCN [mM]	Acid/amide ratio
10	30	1.3
20	60	1.3
50	150	2.5
100	300	2.8

25 °C, 250 mM citrate buffer pH 5.0

We previously reported the pH Influence on the acid/amide ratio in PfNLase catalyzed hydrolysis.⁷ Hence, the KCN concentration would also be expected to influence the acid/amide ratio. With starting concentrations ≤ 20 mM **1** the acid/amide ratio remained unchanged (see Table 2), as would be expected because the pH did not shift. Severe shifts of the acid/amide ratio were observed at starting concentration of 50 and 100 mM **1** but surprisingly these are opposite to what was observed previously.⁷ It would seem that the high acid/amide in these latter experiments is not a pH effect but could be ascribed to selective inhibition by KCN.

2.2. Conversions in biphasic medium

From the experiments described in the subchapter 2.1 we concluded that adoption of a biphasic reaction medium was required to use HCN rather than KCN as the cyanide donor, obviating the disastrous pH shift. A biphasic reaction system moreover will also separate the reactants from contact with the biocatalyst, which could additionally improve the cells stability. DIPE was adopted as the organic solvent, although it is not ideal with regard to the stability of *E. coli*, because it dissolves HCN at high concentrations. Reactions were carried out starting with 10 – 200 mM **1** and HCN in a biphasic system consisting of 30% 50 mM citrate buffer pH 5.0 and 70% DIPE and the reaction progress was measured (Figure 4).

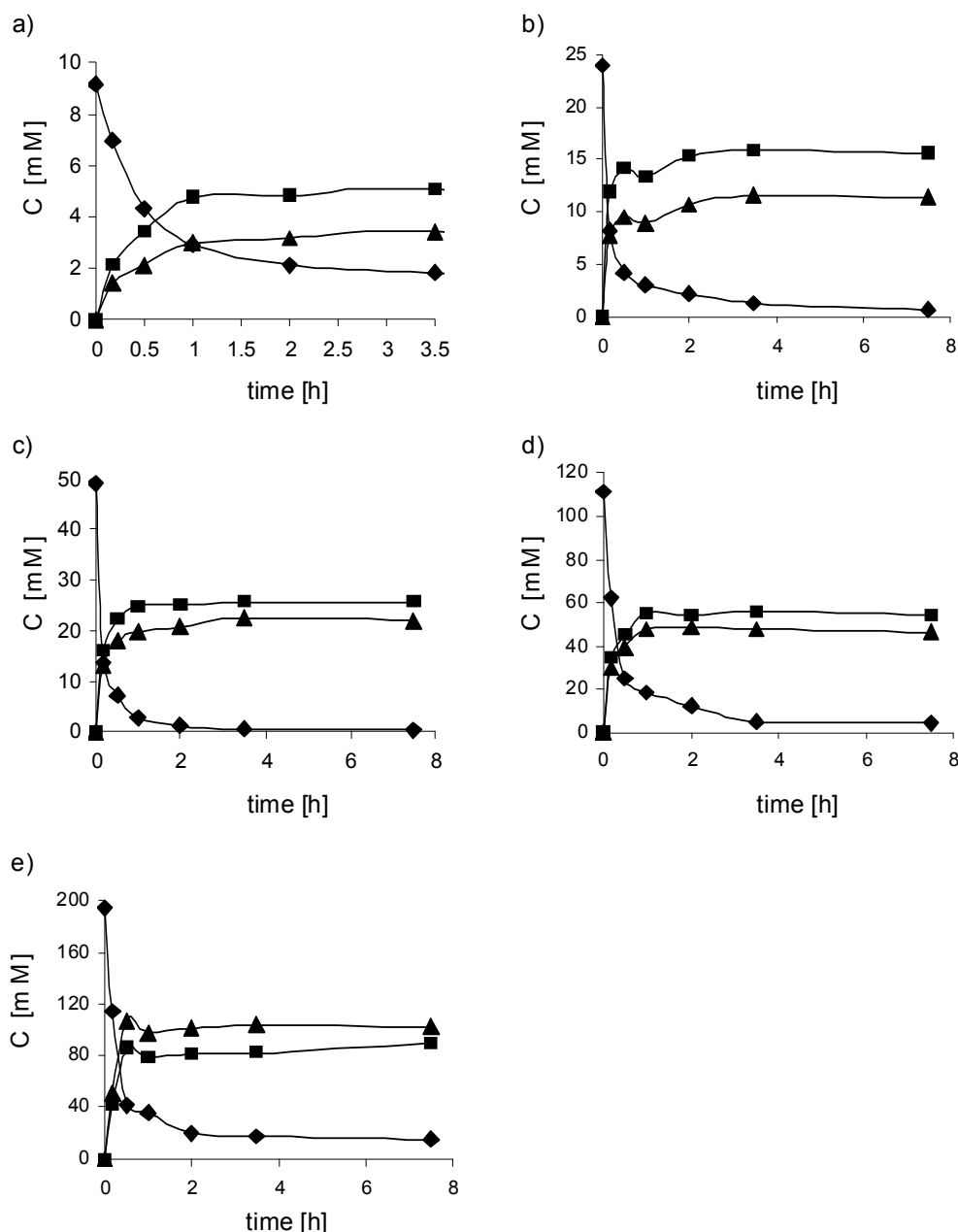


Figure 4. One-cell, *E. coli* expressed (S)-HnL and PfNLase in conversions of **1** (10 – 100 mM) and HCN (30 – 600 mM), a) – e) respectively, into (S)-**2** in bi-phasic reaction consisting on 30 % (v/v) 50 mM citrate buffer at pH 5.0 and 70 % (v/v) DIPE at 25 °C. **1** (◆), **2** (■), **3** (▲).

All the reactions, except the one starting at 10 mM **1**, which did not reach completion due to HCN escape, were completed in less than 3.5 h. Unlike in the monophasic reactions, the biphasic system allowed synthesis of (S)-**2** with excellent enantiopurity ($ee(S) > 99\%$). No pH change was observed. Also in these reaction series, a change in acid/amide ratio, depending on the starting concentrations, was observed (Table 3).

Table 3. The change of acid/amide ratio in one-pot and two step synthesis of (S)-**2** using one-cell *E. coli* expressed (S)-HnL and PfNLase in bi-phasic system.

1 [mM]	HCN [mM]	Acid/amide ratio
10	30	1.50
25	75	1.41
50	150	1.25
100	300	1.14
200	600	0.80

25 °C, 30 % (v/v) 50 mM citrate buffer pH 5.0 and 70 % (v/v) DIPE

Consequently, unlike in the reactions with KCN, here increasing the reactant concentrations stimulated PfNLase to generate more amide and in the reaction with 200 mM **1**, the (S)-**3** concentration exceeded that of (S)-**2** (0.8 ratio). As shown in Chapter 6, the HCN concentration has an effect on the acid/amide ratio.

Further improvements in increasing the reaction rates we believe, could be achieved by changing the organic solvent into one with a higher log P value. We tried to substitute DIPE with e.g. *n*-heptane (log P 4.0). Unfortunately the test failed as it was not possible to extract HCN into this latter solvent.

3. Conclusions

The results shown in this Chapter demonstrate that it is possible to simultaneously express a plant derived HnL and a bacterial NLase in *E. coli* and that the double clone cells are efficient catalysts for synthesizing (S)-**2**. The catalytic efficiency of the double clone is better in aqueous buffer than in an aqueous-organic biphasic system but, when applying KCN as the cyanide donor, pH control is rapidly lost and the resulting pH shift towards alkaline defeats the whole purpose of the bienzymatic procedure. The biphasic system, in contrast, although slower, is much more suitable in reactions with high concentration of reactants.

A strong dependence of the acid/amide ratio on the reactant concentration and pH was observed. This latter phenomenon might open up a more general way for the

enantioselective synthesis of α -substituted amides, which is currently hampered by the generally observed limited enantioselectivity of nitrile hydratases.

4. Materials and methods

Chemicals

Benzaldehyde, 1,3-dimethoxy benzene, di-isopropyl ether, KCN, (*R,S*)-mandelic amide and (*R,S*)-mandelic acid were obtained from Acros, Sigma-Aldrich, Fluka or Lancaster and were used as delivered without further purification. HCN solution in di-isopropyl ether was prepared as described before.⁸

Analytical methods

The conversions of benzaldehyde, mandelic acid and mandelic amide were monitored by reversed phase HPLC on a 4 × 125 mm 5 μ m Lichrospher-RP8 column (Trentec, Gerlingen, Germany) and a Waters Diode Array Detector G1315B set at 215 nm. Mobile phase H₂O-MeOH 60:40 + 0.3% (v/v) H₃PO₄ at 1.0 mL·min⁻¹.

Enantiomeric excess measurements: Chiral HPLC using a ChromTech Chiral-HSA 4 × 150 column (Chromotech AB, Hägerstern) and a Waters Diode Array Detector G1315B set at 215 nm. Eluant; 100 mM phosphate buffer pH 7 at 0.65 mL·min⁻¹.

Experiments

Mono-phasic: All the conversions were carried out in 2 mL plastic eppendorf tubes. Into 250 mM citrate buffer pH 5.0, the cells (OD_{546 nm} 1.6), IS (10 mM), KCN (30, 75, 150, 300 and 600 mM) and benzaldehyde (10, 25, 50, 100 and 200 mM) were added. The final reaction volume was 1.0 mL. The reactions were shaken on an orbital shaker at 1200 rpm and 25 °C. Samples were taken periodically and analyzed by HPLC.

Bi-phasic: All the conversions were carried out in a 2 mL plastic eppendorf tubes. Into 50 mM citrate buffer pH 5.0, the cells (OD_{546 nm} 1.8), IS (10 mM), HCN (30, 60, 150, 300 and 600 mM) and benzaldehyde (10, 20, 50, 100 and 200 mM) were added. The final reaction volume was 1.0 mL. The reactions were shaken on an orbital shaker at 1200 rpm at 25 °C. Samples were taken periodically and analyzed by HPLC.

5. Reference list

1. Cohen, S. N.; Chang, A. C. Y.; Boyer, H. W.; Helling, R. B. *Proc. Nat. Acad. Sci. USA* **1973**, *70*, 3240-3244.
2. Sosedov, O.; Matzer, K.; Bürger, S.; Kiziak, C.; Baum, S.; Altenbuchner, J.; Chmura, A.; Rantwijk, F. v.; Stolz, A. *Adv. Synth. Catal.* **2009**.
3. Rustler, S.; Motejadded, H.; Altenbuchner, J.; Stolz, A. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 87-97.
4. Heipieper, H. J.; Weber, F. J.; Sikkema, J.; Keweloh, H.; De Bont, J. A. M. *Trends Biotechnol.* **1994**, *12*, 409-415.
5. Aono, R.; Kobayashi, H.; Joblin, K. N.; Horikoshi, K. *Biosci. Biotech. Biochem.* **1994**, *58*, 2009-2014.
6. Rajagopal, A. N. *Enzyme Microb. Technol.* **1996**, *19*, 606-613.
7. Fernandes, B. C. M.; Mateo, C.; Kiziak, C.; Chmura, A.; Wacker, J.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 2597-2603.
8. Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1655-1661.

Nitrile Hydratase Activity of a Recombinant Nitrilase*

6

The Chapter contents have been published in:

Fernandes, B. C. M.; Mateo, C.; Kiziak, C.; Chmura, A.; Wacker, J.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, 348, 2597-2603.

* The work described in this Chapter was performed in collaboration with B.C.M. Fernandes.

1. Introduction

It is generally accepted that Nature employs two enzymatic pathways for nitrile hydrolysis (see Figure 1).¹ In the direct pathway a nitrilase (NLase, E.C. 3.5.5.1) catalyzes the addition of two molecules of water to give the carboxylic acid. NLases are widely found in Nature and have been isolated from microbes and plants. The second pathway is mainly present in microbes and involves a hydration step in the presence of a nitrile hydratase (NHase, E.C. 4.2.1.84), which yields the corresponding amide, followed by amidase-catalyzed hydrolysis of the latter.

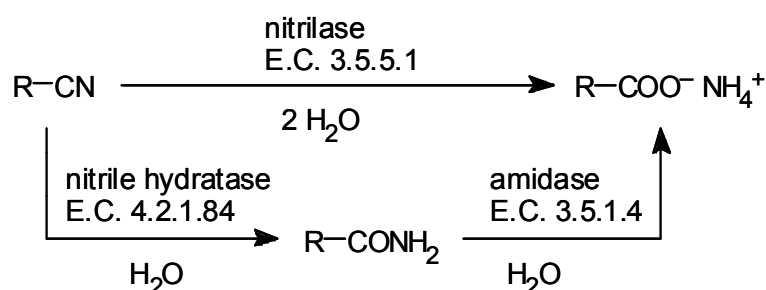


Figure 1. Pathways of enzymatic nitrile hydrolysis.

In contrast with common wisdom, which dictates that NLases transform nitriles into the carboxylic acids exclusively, reports can be found, scattered in literature as far back as the 1960's, of modest amounts of amides being formed in the presence of NLases.²⁻⁴ This side-activity, which is not accounted for by the commonly accepted NLase mechanism,³ has largely been neglected. Presumably, amide formation often was ascribed to the presence of a contaminating NHase. In many cases, amide formation may have gone unnoticed, either because a contaminating amidase hydrolyzed the amide by-product into the acid or because the detection of the catalytic activity or the selection of catalysts depended on ammonia release, which only occurs upon full hydrolysis into the acid.

In a recent characterization study of a purified, recombinantly expressed NLase from *Arabidopsis thaliana* (AtNIT4), > 60% amide was formed in the course of the hydrolysis of 3-cyano-L-alanine, the presumed natural substrate.⁵ A different recombinant NLase from *A. thaliana* (AtNIT1) has been the subject of the most complete study up to date, which showed the effects of electron-withdrawing

substituents on amide formation.^{6, 7} Thus, hydrolysis of 2-crotononitrile in the presence of AtNIT1 afforded 99% crotonic acid, whereas 95% amide was formed from the electron-deficient 3-nitroacrylonitrile.⁷ It is worth noting in this respect that the cysteine-dependent protease papain has been transformed, by changing one amino acid residue, into a modestly active NHase.⁸ We hypothesize, on the basis of the experimental evidence, that the NLase and NHase activities in NLases are intimately related.

In previous Chapters (Chapter 3, 4 and 5) we reported that appreciable amounts of amide are formed in the course of nitrile hydrolysis in the presence of a recombinant NLase originating from *Pseudomonas fluorescens* EBC 191 (PfNLase), depending on the α -substituent and the reaction conditions. The synthetic relevance of amide formation in the presence of NLases prompted us to study this latter side-activity in more detail, to get a better understanding of the factors that influence the amide formation, such as steric requirements and electron density. For this purpose we employed PfNLase, which is known to be prone to amide formation.⁹ It is worth noting that this latter enzyme is an arylacetone nitrilase and tolerates quite bulky groups on the α -position, in contrast to the NLases from *A. thaliana*. The use of an enzyme expressed in *E. coli* ensures that a single nitrile hydrolysing enzyme is present as the non-transformed *E. coli* strain shows no nitrile hydrolysing activity.

In this Chapter we will present the results of our studies on NLase-catalyzed amide formation. The topic deserves to be studied in detail because it may have serious implications regarding the yield and purity of the desired product and complicates the downstream processing. Furthermore, highly selective NLase-mediated amide synthesis, assuming that this latter activity can be controlled, would afford a synthetic route to enantiomerically pure amides. As such, it could be a valuable addition to the synthetic repertoire as NLases are often enantioselective, whereas it is an exception with NHases.

2. Results and discussion

2.1. Hydrolysis of 2-phenylacetonitrile

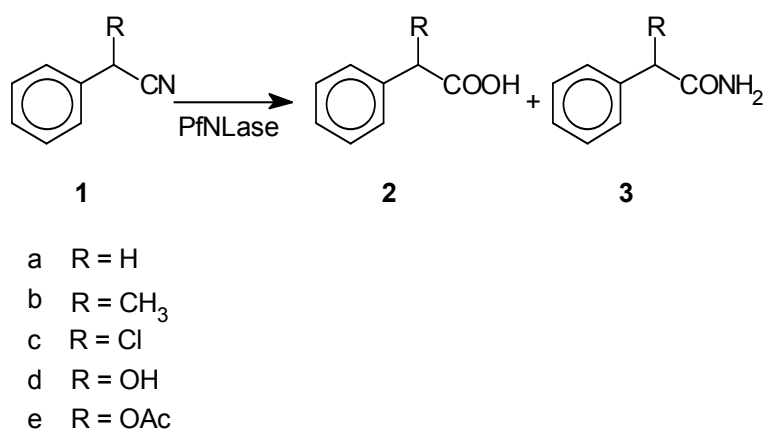


Figure 2. Hydrolysis of nitriles using a nitrilase.

Hydrolysis of 2-phenylacetonitrile (**1a**, Figure 2), a simple model substrate, in the presence of PfNLase afforded, besides 2-phenylacetic acid (**2a**), a minor amount of 2-phenylacetamide (**3a**). From the time-course of the reaction (Figure 3), it becomes apparent that **2a** and **3a** are formed concurrently. Temporary accumulation of amide and subsequent hydrolysis are not observed. Hence, **3a** is not an intermediate in the hydrolysis of **1a** into **2a**, as with nitrile hydratase/amidase systems. It is pertinent to note that PfNLase does not hydrolyze **3a**, neither does it mediate the formation of **3a** from **2a** and ammonia.

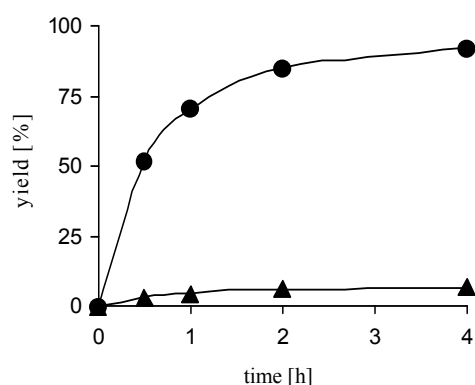


Figure 3. Hydrolysis of 2-phenylacetonitrile (10 mM) in the presence of PfNLase (35.5 $\mu\text{g/mL}$) in 100 mM phosphate buffer pH 6 at 10 $^{\circ}\text{C}$; acid (●), amide (▲).

Summarizing, amide and acid are both *bona fide* reaction products that are formed directly from the nitrile in a ratio of 23:1. This latter parameter, the acid/amide ratio (Ac/Am), will be used throughout the Chapter to characterize the selectivity of PfNLase.

We found that the reaction pH, when varied from 5 to 9, influenced the acid/amide ratio only slightly (Figure 4a), with lower pH slightly reducing the amount of amide. The effect of the reaction temperature was more pronounced (Figure 4b) and Ac/Am changed from 11 at 5 $^{\circ}\text{C}$ to 38 at 40 $^{\circ}\text{C}$. Measurements of the initial rates of formation of **2a** and **3a** (data not shown) showed that acid formation is more temperature-dependent, which indicates a higher activation barrier for the formation of acid. These results suggest that elevated temperature and low pH steer the reaction towards acid, whereas a low temperature and increased pH bend the selectivity towards the amide.

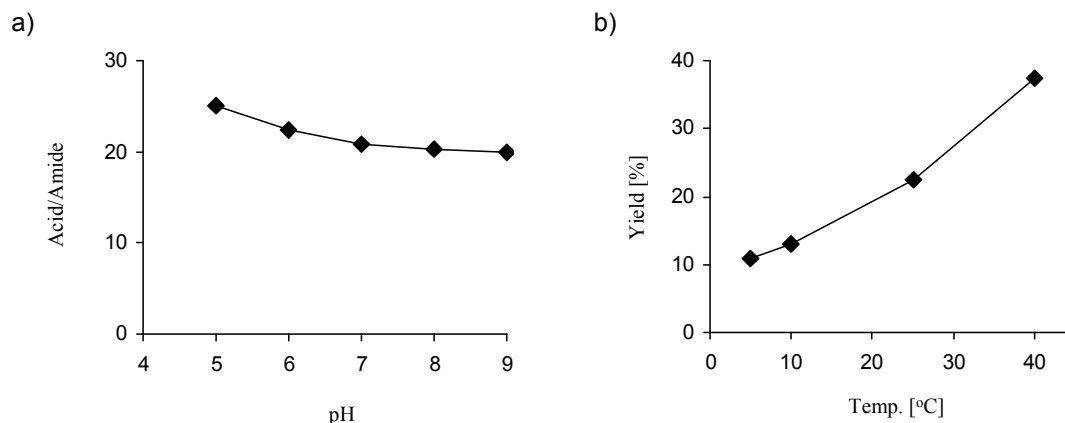


Figure 4. Acid/Amide ratio for PfNLase (35.5 $\mu\text{g/mL}$) in the hydrolysis of 2-phenylacetonitrile (10 mM) in 100 mM phosphate buffer; effect of pH at 25 $^{\circ}\text{C}$ (a); temperature effect at pH 6 (b).

2.2. Electronic effects of the α -substituent

It has been shown that the Ac/Am ratio of a AtNIT1 NLase was highly sensitive to electronic effects.⁷ We wished to investigate the behavior of PfNLase in this respect by substituting the α -position in **1a** with similarly sized groups of opposing electronic character. Thus, we compared 2-phenylpropionitrile (**1b**, see Figure 2) and 2-chloro-2-phenylacetonitrile (**1c**) and found that the electron-deficient **1c** reacts faster by a factor of 2.4.¹⁰ The methyl-substituted reactant **1b** was converted into > 99% 2-phenylpropionic acid (**2b**), whereas 90% amide was formed from **1c** (see Table 1). Hence, it would seem that the extent of amide increases with increasing electronegativity of the α -substituent: $\text{CH}_3 < \text{H} < \text{Cl}$. A comparable effect of α -fluoro substitution has been reported for *Arabidopsis* NLases.⁶

Interestingly, the formation of 2-chloro-2-phenylacetic acid (**2c**) from racemic **1c** was strongly biased towards the (*R*)-enantiomer (Table 1), whereas 2-chloro-2-phenylacetic amide (**3c**) was nearly enantiomerically pure (*S*). This result strongly indicates that the absolute configuration of the α -carbon strongly influences the Ac/Am ratio but, unfortunately, this effect could not be investigated in detail as **1c** racemized under the reaction conditions.

Table 1. Electronic effects on the acid/amide ratio.

	1b	1c
Initial rate [$\mu\text{mol min}^{-1} \text{mg}^{-1}$]	0.81	2.13
Acid [%]	>99	11
ee [%]	n.d.	82 (<i>R</i>)
Amide [%]	<1	89
ee [%]	n.d.	> 94 (<i>S</i>)
Ac/Am	>99	0.1

Reaction conditions: 10 mM nitrile in 100 mM phosphate buffer pH 6 at 25 °C.

2.3. Effects of the α -substituent size and the absolute configuration

Enantiomerically pure mandelic acid (**2d**) is industrially produced as a chiral intermediate in the synthesis of fine chemicals and as a chiral auxiliary in industrial resolutions. (*R*)-**2d** is produced ¹¹ on a multiton scale via enantioselective hydrolysis of mandelonitrile (**1d**) in the presence of a nitrilase.¹² The pure enantiomers of **1d** are readily accessible via hydrocyanation of benzaldehyde in the presence of the appropriate oxynitrilase;¹³ hence, they are, in principle, good model compounds for investigating possible effects of the absolute configuration of the reactant on the Ac/Am ratio.

The modest stability of **1d** in neutral aqueous medium is an obstacle that requires careful consideration. As explained in Chapter 2, compound **1d** decomposes into benzaldehyde and hydrogen cyanide; the process is reversible and is the major racemisation pathway of enantiomerically pure **1d**. The nitrile becomes stable at pH < 4 – 5 and lower temperature but such conditions also have a strongly deleterious effect on nitrilase activity. We compromised by investigating the hydrolysis of enantiomerically pure (*R*)- and (*S*)-**1d** in the presence of PfNLase at pH 6 and 0 °C. Under these conditions the racemisation of the nitrile is much slower than the enzymatic hydrolysis. Both enantiomers reacted with comparable rates, but with widely diverging product selectivity (Table 2). While the hydrolysis of (*R*)-**1d** yielded only 11% amide (Ac/Am 8.1), the amide (*S*)-**3d** was the major product from the

hydrolysis of (*S*)-**1d** (Ac/Am = 0.8). Hence, it appears that Ac/Am is influenced by electronic as well as by steric factors. Here, the study of enantiopure compounds gives an insight into the system that is never possible by the use of racemates.

A more stable derivative of **1d** was required to measure the effects of the pH on amide formation. The ester derivative, 2-acetoxy-2-phenylacetonitrile (**1e**),^{14, 15} does not suffer from HCN elimination but racemisation still occurs, *via* deprotonation on the α -position. Fortunately, this latter process is slow in aqueous media at pH <7 (data not shown). Spontaneous hydrolysis of the ester bond in **1e** (with formation of **2d** and **3d**), which is significant at pH >7, remained restricted to <5% but often much less, depending on the pH.

Table 2. Hydrolysis of mandelonitrile and 2-acetoxy-2-phenylacetonitrile in the presence of PfNLase.

	(<i>R</i>)- 1d	(<i>S</i>)- 1d	(<i>R</i>)- 1e	(<i>S</i>)- 1e
Initial rate [$\mu\text{mol min}^{-1} \text{mg}^{-1}$]	0.62	0.62	0.23	1.73
Acid [%]	89	45	67	38
Amide [%]	11	55	33	62
Ac/Am	8.1	0.8	2	0.6

Reaction conditions: 10 mM reactant, 100 mM phosphate buffer pH 6 at 0 °C (mandelonitrile) or 10% methanol at 25 °C (2-acetoxy-2-phenylacetonitrile).

Much to our surprise, the ester group profoundly influenced the course of the enzymatic hydrolysis. The reaction rates (Table 2) should not be compared directly as different temperatures were used, but it is noteworthy that (*R*)-**1e** reacted three times slower than (*R*)-**1d**, the 25 °C temperature difference notwithstanding. The enantiomeric kinetic bias shifted from pro-(*R*), with **1d**, to pro-(*S*). Both enantiomers produced more amide than the corresponding enantiomers of the free cyanohydrin; the Ac/Am ratio of the hydrolysis of (*R*)-**1e** was even four times lower than that of (*R*)-**1d**. The progress curve for the separate hydrolysis of (*R*)- and (*S*)-**1e** (Figure 5) illustrates well that the amide is not a reaction intermediate and that the two enantiomers are differently hydrolysed.

We conclude, on the basis of these results, that enantiomers of chiral reactants should be treated as separate entities, and that unambiguous conclusions as regards selectivity issues cannot be based on racemate conversions. Thus, for example, in the hydrolysis of (*R,S*)-**1e**, (*S*)-**2e** and (*S*)-**3e** are the major products initially, with $Ac/Am = 0.7$, whereas at total conversion (*R*)-**2e** and (*S*)-**3e** predominate ($Ac/Am=1.1$, data not shown).

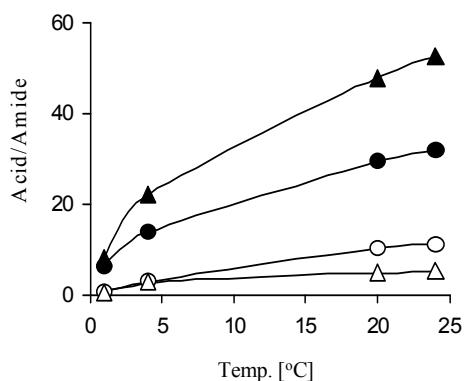


Figure 5. Hydrolysis profiles for (*R*)- and (*S*)-2-acetoxy-2-phenylacetonitrile, (*S*)-2-acetoxy-2-phenylacetic acid (●), (*S*)-2-acetoxy-2-phenylacetic amide (▲), (*R*)-2-acetoxy-2-phenylacetic acid (○), (*R*)-2-acetoxy-2-phenylacetic amide (△) at pH 6.

A pH profile of the hydrolysis of **1e** (Figure 6) revealed widely diverging effects on the propensity of the two enantiomers to produce amide. Amide formation from (*R*)-**1e** was found to increase when the medium was turned acidic, whereas the Ac/Am ratio of the (*S*)-enantiomer passed through a minimum at pH 6. Both patterns are completely different from what is seen with **1a**. Changes in charge distribution obviously affect (*R*)- and (*S*)-**1e** in different ways, which leads us to the conclusion that the enantiomers bind in very different ways.

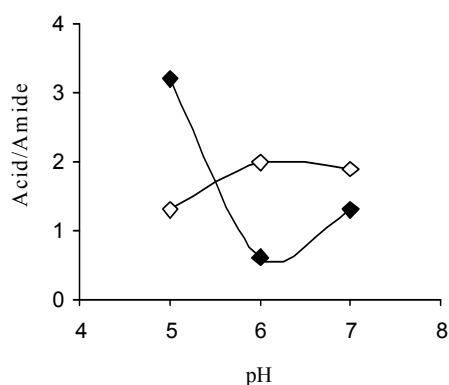
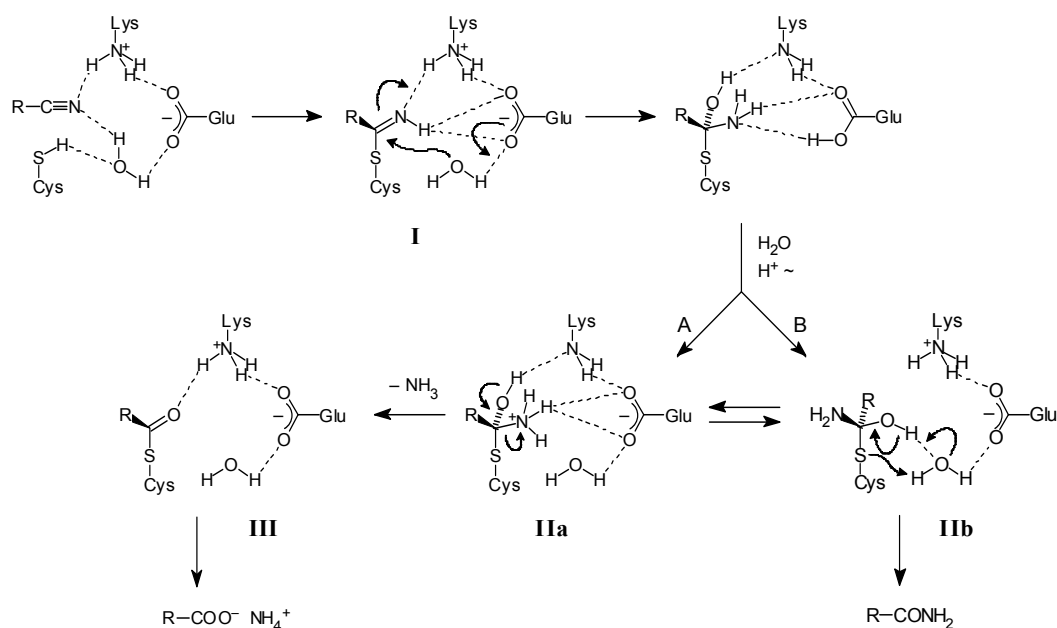


Figure 6. pH profile for the hydrolysis of (*R*)-2-acetoxy-2-phenylacetonitrile (◆) and (*S*)-2-acetoxy-2-phenylacetonitrile (◇); reaction conditions: 10 mM reactant, 100 mM phosphate buffer, 25 °C.

2.4. Mechanism of action

Any discussion of the catalytic mechanism of nitrilases is hampered by a lack of crystal structures. It is commonly accepted, nevertheless, that nitrilases harbor a Cys-Glu-Lys triad in the active site¹⁶ and that the reaction takes place via a thioimide intermediate (I, Scheme 1).^{2, 3, 17} The active site triad has been identified in the crystal structures of the worm NitFhit fusion protein¹⁸ and in a putative nitrile hydrolase from yeast.¹⁹ This nitrile hydrolysis mechanism is depicted in Scheme 1. A bridging water molecule has been added because it has been identified in the yeast protein,¹⁹ as well as in a structurally closely related D-carbamoylase (*N*-carbamoyl-D-amino acid amidohydrolase).^{20, 21}



Scheme 1. Proposed nitrilase mechanism for the formation of acid (A) and amide (B).

Elimination of ammonia from the tetrahedral intermediate (pathway A), with formation of the acyl-enzyme intermediate III, requires a positive charge on the nitrogen atom in the reactant, stabilized by the Glu residue. If, in contrast, the positive charge is not on the reactant but, for example, on the Lys residue (tetrahedral intermediate IIb), formal thiol elimination is expected to prevail (pathway B). Such a charge distribution could be expected, for example, when an electron-demanding R-group destabilizes the positive charge on the reactant N. Otherwise, steric interactions could force the N atom away from the stabilizing Glu (see Scheme 1, pathway B). Summarizing, NLase-mediated nitrile hydrolysis into the carboxylic acid and water addition to give the amide are two branches of the nitrilase mechanism, as originally suggested by Hook and Robinson.² We propose that the charge distribution in the tetrahedral intermediate, depending, in turn, on the stereochemical and electronic properties of the R-group acts as a mechanistic switch.

We note that amide elimination from the tetrahedral intermediate is amply supported in the chemical literature. It is the major pathway in the hydrolysis of thioimide esters²² and mercaptoethanol-catalyzed nitrile hydrolysis²³⁻²⁵ at neutral pH. In both cases the product selectivity shifted towards acid at pH < 3. We found a similar pH effect, although under near-neutral conditions and of smaller magnitude, in the enzymatic hydrolysis of **1a**.

The active site does not contain bound amide at any time, according to our mechanism and amide, once formed, is not hydrolyzed any further. It is commonly observed that NLases do not hydrolyse amides, although there are strong indications that NLases, aliphatic amidases and D-carbamoylases have very similar active site architectures.²⁶ A very modest amidase activity (6000 times less than the NLase activity), mainly due to a reduced $v_{\max}$, has been measured with the nitrilase from *Rhodococcus rhodochrous* J1.^{27, 28} If this factor also applies to PfNLase, hydrolysis of **3e** would be in the order of 0.01% over 24 h.

Why amides cannot react via IIa into the thiol ester intermediate (III) is not clear but an obvious explanation is that IIa is too high in energy, relative to the amide. As regards the amidase from *Pseudomonas aeruginosa*, it has been suggested that only one amide molecule can be bound per hexameric amidase molecule,²⁷ possibly because conformational changes in the entire hexamer are required to smooth the energetic pathway towards the acyl-enzyme intermediate.

As regards nitrilase catalysis, it would seem that the enzyme conserves the chemical energy in the nitrile triple bond, maintaining IIa and IIb well above ground-state level, to transform the reactant smoothly into acid and amide.

3. Conclusions

We have shown that *Pseudomonas fluorescens* nitrilase converts nitriles into the carboxylic acid as well as the amide. The relative formation of acid and amide is subject to the pH and the temperature. Electron-withdrawing substituents at the α -position favor amide formation.

We have shown, for the first time, that the absolute configuration at the α -position in the reactant exerts a dramatic influence on the extent of amide formation.

We have also proposed the possible mechanism that can steer the nitrile to be transformed into either the corresponding acid or amide.

4. Materials and methods

Chemicals

Soluble, His₆-tagged *Pseudomonas fluorescens* EBC 191 nitrilase was recombinantly produced in *E. coli* JM109(pLK9).⁹ The stock solution contained approx. 48 mg mL⁻¹ of protein, according to a Bradford test. Its activity in the hydrolysis of racemic mandelonitrile was 3.1 $\mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$ at 0 °C and pH 6. 2-Phenylpropionitrile from Acros was purified by distillation under reduced pressure in a Kugelrohr apparatus (Büchi) to remove an unknown contaminant that inhibited PfNLase. Unless otherwise stated, the chemicals were obtained from normal commercial sources and used without further purification.

(*R*)- and (*S*)-mandelonitrile were synthesised *via* enzymatic hydrocyanation of benzaldehyde in the presence of the appropriate oxynitrilase.^{29, 30} Enantiomeric purities were verified by chiral HPLC to be 99% (*R*) and 98% (*S*).

(*R*)-2-Acetoxy-2-phenylacetonitrile was synthesised by acylation of (*R*)-mandelonitrile with acetic anhydride and pyridine in 1,4-dioxane at room temperature. The mixture was diluted with ether and consecutively washed with water, 1 M hydrochloric acid, saturated sodium carbonate and brine, dried over MgSO₄ and concentrated *in vacuo*. The product was isolated as a colourless liquid with ee > 99% according to chiral GC. ¹H NMR (300 MHz, CDCl₃): δ = 2.16 (s, 3H, CH₃), 6.41 (s, 1H, CH), 7.26 – 7.55 (m, 5H, aromatic); ¹³C NMR (75 MHz, CDCl₃): δ = 20.4 (CH₃), 62.8 (CH), 11.6 (CN), 127.8 (C-2,6), 129.2 (C-3,5), 130.3 (C-4), 131.7 (C-1), 168.9 (C=O).

(*S*)-2-Acetoxy-2-phenylacetonitrile was obtained using a published procedure³¹ with enantiomeric purity 93.7% according to chiral GC.

Analysis

Conversions and yields were determined by reversed phase HPLC analysis, using a Waters 590 pump, a 50 × 4.6 mm Merck Chromolith SpeedROD RP-18e column and a Waters 486 UV detector. Mobile phase ACN-H₂O-TFA 10:90:0.1 (2-phenylacetonitrile, 2-phenylacetic acid and amide), 20:80:0.1 (2-phenylpropionitrile, 2-chloro-2-phenylacetonitrile, mandelonitrile and 2-acetoxy-2-phenylacetonitrile as well as their hydrolysis products) at 1 mL·min⁻¹.

Enantiomeric purities were determined by chiral HPLC, using a Waters 510 pump, a 4.6 × 250mm Chiracel AD-H column and a Waters 486 UV detector. Mobile phase hexane-isopropyl alcohol-TFA 95:5:0.1 (2-chloro-2-phenylacetic acid and amide), 92:8:0.1 (2-acetoxy-2-phenylacetic acid and amide).

Chiral GC of 2-chloro-2-phenylacetoneitrile and 2-acetoxy-2-phenylacetoneitrile was performed with a Shimadzu GC-17A instrument equipped with a Shimadzu Auto Injector AOC-20i, a 25 m × 0.25 mm Varian CP-Chirasil-Dex CB column and an FID detector. Column temperature: 140 °C (2-chloro-2-phenylacetoneitrile), 145 °C (2-acetoxy-2-phenylacetoneitrile).

Enzymatic hydrolysis

Enzymatic hydrolysis of all the nitriles was carried out at 1-2 mL scale in magnetically stirred Eppendorf tubes immersed in a water-bath at the desired temperature. Stock solutions of the substrate and internal standard (in methanol), PfNLase and buffer (in water) were used. Final concentrations were 10 mM of substrate, 1mM internal standard, 10% methanol and 100 mM phosphate buffer at the desired pH.

The reaction was started by the addition of the enzyme solution (all enzyme weights refer to the total protein content). Samples were taken periodically, diluted with acid (to stop the reaction) and filtered over a Microcon YM-10 centrifugal filter device prior to analysis. Samples for chiral analysis were further extracted with ether and dried over MgSO₄. The solvent was evaporated and residue was redissolved in the appropriate solvent.

Reported Ac/Am ratios were measured when these had stabilized and stayed constant until full conversion.

5. Reference list

1. Nagasawa, T.; Yamada, H. *Trends Biotechnol.* **1989**, *7*, 153-159.
2. Hook, R. H.; Robinson, W. G. *J. Biol. Chem.* **1964**, *239*, 4263-4267.
3. Goldlust, A.; Bohak, Z. *Biotechnol. Appl. Biochem.* **1989**, *11*, 581-601.
4. Stevenson, D. E.; Feng, R.; Dumas, F.; Groleau, D.; Mihoc, A.; Storer, A. C. *Biotechnol. Appl. Biochem.* **1992**, *15*, 283-302.

5. Piotrowski, M.; Schonfelder, S.; Weiler, W. *J. Biol. Chem.* **2001**, 276, 2616-2621.
6. Effenberger, F.; Osswald, S. *Tetrahedron: Asymmetry* **2001**, 12, 279-285.
7. Osswald, S.; Wajant, H.; Effenberger, F. *Eur. J. Biochem.* **2002**, 269, 680-687.
8. Dufour, E.; Storer, A. C.; Menard, R. *Biochemistry* **1995**, 34, 16382-16388.
9. Kiziak, C.; Conradt, D.; Stolz, A.; Mattes, R.; Klein, J. *Microbiology-Sgm* **2005**, 151, 3639-3648.
10. Electron-deficiency in esters is known to increase their reactivity towards lipases, which likewise act via general base catalysis.
11. Breuer, M.; Ditrich, K.; Habicher, T.; Hauer, B.; Kessler, M.; Sturmer, R.; Zelinski, T. *Angew. Chem., Int. Ed.* **2004**, 43, 788-824.
12. Yamamoto, K.; Oishi, K.; Fujimatsu, I.; Komatsu, K. I. *Appl. Environ. Microbiol.* **1991**, 57, 3028-3032.
13. Schmidt, M.; Griengl, H. In *Biocatalysis- From Discovery to Application*; Fessner, W.-D. Ed. Oxynitrilases: From Cyanogenesis to Asymmetric Synthesis. Springer: 1999; pp. 194-226.
14. Layh, N.; Stolz, A.; Forster, S.; Effenberger, F.; Knackmuss, H. J. *Arch. Microbiol.* **1992**, 158, 405-411.
15. Heinemann, U.; Kiziak, C.; Zibek, S.; Layh, N.; Schmidt, M.; Griengl, H.; Stolz, A. *Appl. Microbiol. Biotechnol.* **2003**, 63, 274-281.
16. Brenner, C. *Curr. Opin. Struct. Biol.* **2002**, 12, 775-782.
17. Harper, D. B. *Biochem. J.* **1977**, 165, 309-319.
18. Pace, H. C.; Hodawadekar, S. C.; Draganescu, A.; Huang, J.; Bieganowski, P.; Pekarsky, Y.; Croce, C. M.; Brenner, C. *Curr. Biol.* **2000**, 10, 907-917.
19. Kumaran, D.; Eswaramoorthy, S.; Gerchman, S. E.; Kycia, H.; Studier, F. W.; Swaminathan, S. *Proteins* **2003**, 52, 283-291.
20. Nakai, T.; Hasegawa, T.; Yamashita, E.; Yamamoto, M.; Kumasaka, T.; Ueki, T.; Nanba, H.; Ikenaka, Y.; Takahashi, S.; Sato, M.; Tsukihara, T. *Structure* **2000**, 8, 729-737.
21. Wang, W. C.; Hsu, W. H.; Chien, F. T.; Chen, C. Y. *J. Mol. Biol.* **2001**, 306, 251-261.
22. Chaturvedi, R. K.; MacMahon, A. E.; Schmir, G. I. *J. Am. Chem. Soc.* **1967**, 89, 6984-6993.
23. Zervos, C.; Cordes, E. H. *J. Org. Chem.* **1971**, 36, 1661-1667.
24. Winkler, M.; Glieder, A.; Klempier, N. *Chem. Commun. (Cambridge)* **2006**, 1298-1300.
25. Dithiothreitol-catalyzed amide formation²⁴ may have increased the confusion in the past. The enzyme preparation that we used did not contain dithiothreitol. 200.

26. Pace, H. C.; Brenner, C. *Genome Biol.* **2001**, 2, 1-9.
27. Novo, C.; Farnaud, S.; Tata, R.; Clemente, A.; Brown, P. R. *Biochem. J.* **2002**, 365, 731-738.
28. Kobayashi, M.; Goda, M.; Shimizu, S. *Biochem. Biophys. Res. Commun.* **1998**, 253, 662-666.
29. van Langen, L. M.; Selassa, R. P.; van Rantwijk, F.; Sheldon, R. A. *Org. Lett.* **2004**, 7, 327-329.
30. Forster, S.; Roos, J.; Effenberger, F.; Wajant, H.; Sprauer, A. *Angew. Chem.-Int. Ed. Engl.* **1996**, 35, 437-439.
31. Veum, L.; Kanerva, L. T.; Halling, P. J.; Maschmeyer, T.; Hanefeld, U. *Adv. Synth. Catal.* **2005**, 347, 1015-1021.

**Characterization of a Nitrilase Derived
from a Haloalkaliphilic Gammaproteobacterium
Halomonas nitrilicus sp. nov.**

7

A part of this Chapter contents have been published in:

Chmura, A.; Shapovalova, A.A.; van Pelt, S.; van Rantwijk, F.; Tourova, T.P.; Muyzer, G.; Sorokin
D.Yu. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 371–378.

1. Introduction

Until recently all known nitrile degrading microorganisms were neutrophilic, and there was no evidence of the possibility of nitrile biodegradation at high pH/salt conditions. In 2007 Sorokin *et al.* published the discovery, enrichment and isolation of a new alkaliphilic bacteria capable of growth with a range of aliphatic nitriles as sole C and N source at extremely haloalkaline conditions.^{1, 2} The soda lakes and soda soils in south-eastern Siberia (Russia) are characterized by pH values up to 11 and salt concentrations up to saturation. The high pH values are maintained due to the high buffering capacity of sodium carbonate/bicarbonate, which are among the major anions in the solution. Despite these extreme conditions, soda lakes are highly productive and harbor diverse microbial communities responsible for element cycling.^{3, 4} During the last decades many new species of haloalkaliphilic bacteria belonging to various phylogenetic lineages have been isolated from such an environment. The gammaproteobacterial genus *Halomonas* is one of them. It tolerates wide range of salinity and alkalinity and is highly versatile in terms of ability to utilize various organic compounds, including simple aromatics.

In the previous Chapters the application of a nitrilase (NLase) in biocatalysts composed of two or three enzymes were described (Chapters 3 and 4 respectively). The NLase used in those experiments and many others have an undesired double activity (NLase and nitrile hydratase) that in nitrile hydrolysis results in the formation of carboxylic acids as well as amides. A solution to overcome this latter problem is demonstrated in Chapter 4, where the concept of using an amidase together with the NLase to convert the unwanted amide was introduced. Other solutions would be to reach for the genetic tools to modify the NLase or, alternatively, to screen for NLases that do not produce amides.

In this Chapter the characterization of an NLase derived from haloalkaliphilic *Halomonas nitrilicus* sp. nov (HnNLase) is reported. The *H. nitrilicus* originates from highly alkaline soil surrounding a soda lake in eastern Russia and was selected and enriched on account of its ability to utilize arylaliphatic nitrile as N source. A whole-cell preparation was studied with regards to NLase and nitrile hydratase activity, storage stability and substrate spectrum as well as enantioselectivity. The biocatalyst

was also examined for possible use at high (> 0.1 M) reactant concentrations. Finally, attempts to obtain a cell-free extract of the NLase will be described.

2. Results and discussion

2.1. Growth of *H. nitrilicus* sp. strain ANL- α CH₃, activity and stability of the resting cells

The previously enriched *H. nitrilicus* strain⁶ was grown in sodium carbonate medium strongly buffered at pH 10 with (*R,S*)-2-phenylpropionitrile (**1**) as nitrogen source and sodium acetate as the sole carbon source. After inoculation, **1** was added in portions (at 0 and 12 h from inoculation) and the growth parameters (optical density and nitrile utilization) were monitored (Figure 1).

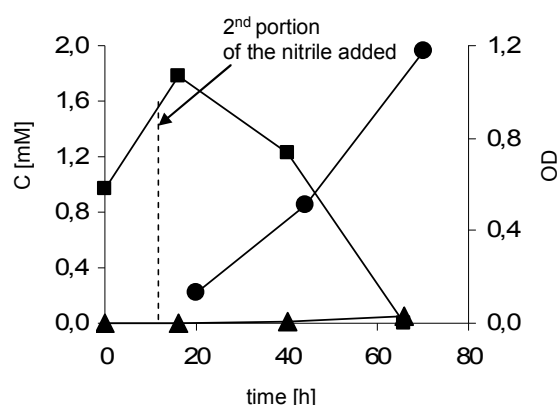


Figure 1. Growth of *H. nitrilicus* sp. bacterium in sodium carbonate medium at pH 10 and 30 °C. (*R,S*)-2-**1** (■), 2-phenylpropionic acid (▲), cell density (●).

Growth did not start immediately. The concentration of **1** after 16 h (after the second **1** portion was added) was 1.8 mM. This indicates that no nitrile was utilized during the first 16 h of the culture growth. After 66 h, all the nitrile was consumed and a non stoichiometric amount (0.05 mM) of the acid was obtained. The cells were harvested by centrifugation and resuspended in sodium carbonate buffer pH 10. The fresh, resting cell suspension contained 5.8 mg protein mL⁻¹. Assays with three different nitriles: **1**, phenylacetoneitrile (**2**), (*R,S*)-mandelonitrile ((*R,S*)-**3**) showed that HnNLase contained an active nitrile degrading enzyme. The hydrolysis rates with the above

nitriles were $0.17 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ((*R,S*)-**3**), $0.05 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (**1**) and $0.72 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (**2**), respectively (see Table 1).

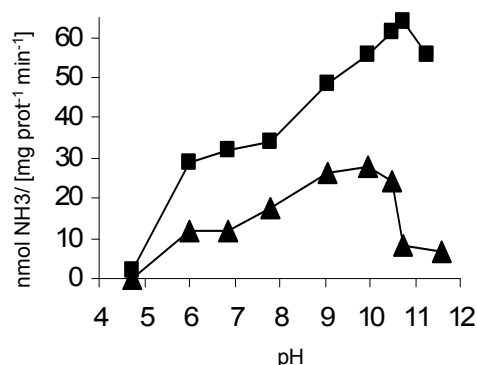


Figure 2. Influence of pH on the activity of HnNLase measured at 30 °C. Acrylonitrile (■), (*R,S*)-2-phenylpropionitrile (▲).

The HnNLase resting cells were active over a wide pH range. In its lower part, the cells were able to convert both substrates already at pH 5. The activity of the cells increased with the alkalinity of the reaction medium until pH 10 (**1**) and pH 10.5 for acrylonitrile (**4**), where the activity maximum was found.

The initial hydrolysis rate of **2** measured after harvesting the cells was $0.7 \mu\text{mol min}^{-1} \text{mg}^{-1}$. The resting cells were stored on ice in sodium carbonate buffer at pH 10. Samples from the ice stored batch were taken periodically to monitor the activity during storage (Figure 3).

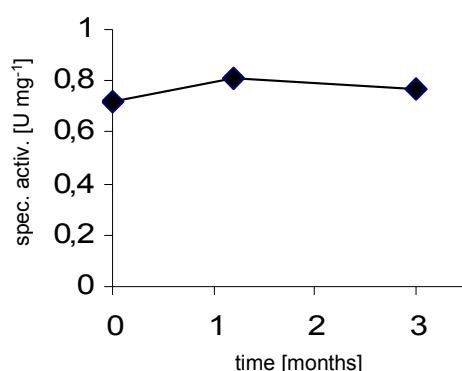


Figure 3. Storage stability of HnNLase resting cells at 0 °C (ice), upon resuspension in sodium carbonate buffer pH 10.

According to the experimental data, the resting cells exhibit excellent stability while stored on ice without any stabilizing agents. There was no activity loss observed even 3 months after harvesting the cells.

2.2. Nitrilase or nitrile hydratase / amidase?

In Chapter 1 the two known enzymatic nitrile degradation pathways that lead to carboxylic acids have been described. Nitriles can be directly hydrolyzed by NLase into the corresponding carboxylic acid ammonium salt. In the other pathway, the nitriles are first enzymatically hydrated to form the corresponding amides, which are subsequently hydrolyzed by an amidase into the carboxylic acids (Chapter 1, Figure 14) When in the two-enzyme nitrile degradation pathway, the second step, amide hydrolysis, is faster than nitrile hydration, no amide will accumulate and, hence, it will escape detection. The goal of the following experiments was to prove that HnNLase hydrolyzes nitriles *via* the NLase pathway.

2.2.1. Incubation of HnNLase resting cells with aromatic amides

In the following experiments the presence of amidase in HnNLase cells was checked by incubation with two different aromatic amides, mandelic amide and 2-phenylpropionic amide (Figure 4). In neither case was there any acid formed after 20 h of the incubation. Hence, we conclude that HnNLase hydrolyzes nitriles exclusively *via* the NLase pathway.

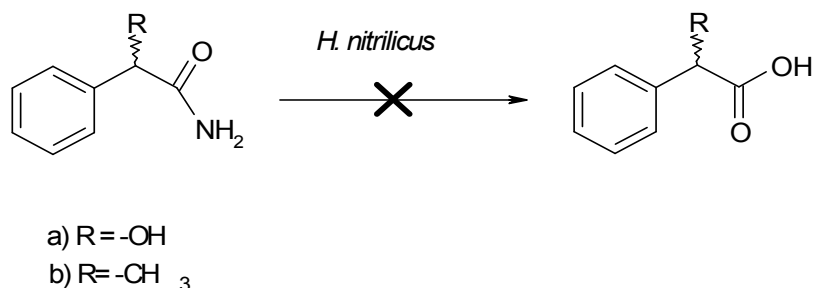


Figure 4. General scheme for the amidase activity check in the HnNLase resting cells.

2.2.2. Growth experiment with phenylacetoneitrile. Metabolism of *H. nitrilicus*

HnNLase can also grow on pure **2** as the only C, N and energy source (Figure 5). During growth, **2** was rapidly metabolized, releasing nearly stoichiometric amounts of ammonia during the early logarithmic growth phase. Only a little intermediate accumulation of phenylacetic acid was observed, however. The biomass production continued after all **2** had disappeared, indicating formation of an essential intermediate. HPLC analysis, however, did not show any formation of phenylacetamide.

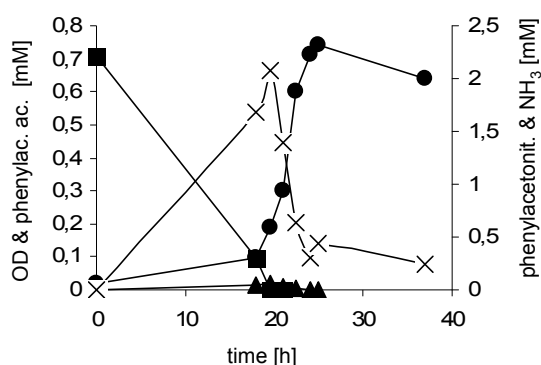


Figure 5. Growth of HnNLase bacterium with phenylacetoneitrile as N, C and energy source in the growth media buffer at pH 10 and 30 °C. Phenylacetoneitrile (■), phenylacetic acid (▲), cell density (●), NH₃ (×).

The high growth yield indicated complete utilization of **2**, which implicates an ability to utilize aromatic carbon, which is not an ordinary property of the genus *Halomonas*. In fact, it is possible that in the metabolic pathway the cells of HnNLase will reduce phenylacetic acid or other carboxylic acid to phenol. The potential to use phenol by the cells at haloalkaline conditions has so far been shown only for a single *Halomonas* species, *H. campisalis*,⁷ – the immediate phylogenetic relative of *H. nitrilicus*. The capacity of *H. nitrilicus* to metabolize aromatic C was confirmed in growth experiments with benzoic and salicylic acids.⁶

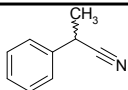
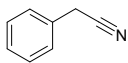
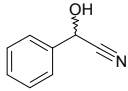
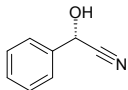
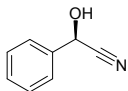
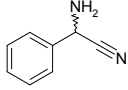
2.3. Substrate spectrum of HnNLase

To characterize the HnNLase, its substrate, enantio- and regioselectivity was examined. Nine different aliphatic and aromatic nitriles with straight and branched

side-chains were screened. Also, the influence of the different α -substituents (and enantiomers) on the reaction rate was investigated (Table 1).

The hydrolyses of (*R,S*)-**3**, (*R*)-**3** and (*S*)-**3** were carried out at pH 5.5 and at low temperature. The rather low pH, just on the lower limit of HnNLase activity and the low temperature were chosen to suppress the decomposition and/or racemization of the cyanohydrins.

Table 1. The substrate and enantiomeric preferences of HnNLase.

Substrate no.	Substrate	Rate [$\mu\text{mol min}^{-1} \text{mg}^{-1}$]	pH
1		0.05	7
2		0.77	7
(<i>R,S</i>)-3		0.18	5.5
		0.18	7.0
		0.026	5.5 (5 °C)
(<i>S</i>)-3		0.0025	5.5 (5 °C)
(<i>R</i>)-3		0.05	5.5 (5 °C)
4		0.21	7
5		$1.1 \cdot 10^{-4}$	7
6		0.0017	7
7		0.46	8.4

Reaction temperature was 25 °C (if not stated otherwise)

Other reaction conditions as specified in materials and methods part

When comparing the experimental data of (*R,S*)-**3** hydrolysis surprisingly, no differences in the initial reaction rates between pH 5.5, and pH 7.0 at 25 °C were

measured. When the reaction temperature in the hydrolysis of (*R,S*)-**3** was lowered to 5 °C, the initial reaction rate dropped seven times compared to the rate measured at 25 °C. The data regarding the racemate of **3** and its pure (*R*)- and (*S*)- enantiomers, presented in Table 1, showed that under the same conditions (temperature, pH) the nitriles are converted with different rates. Further hydrolysis experiments showed that indeed the enzyme is highly enantioselective (Figure 6 a – c).

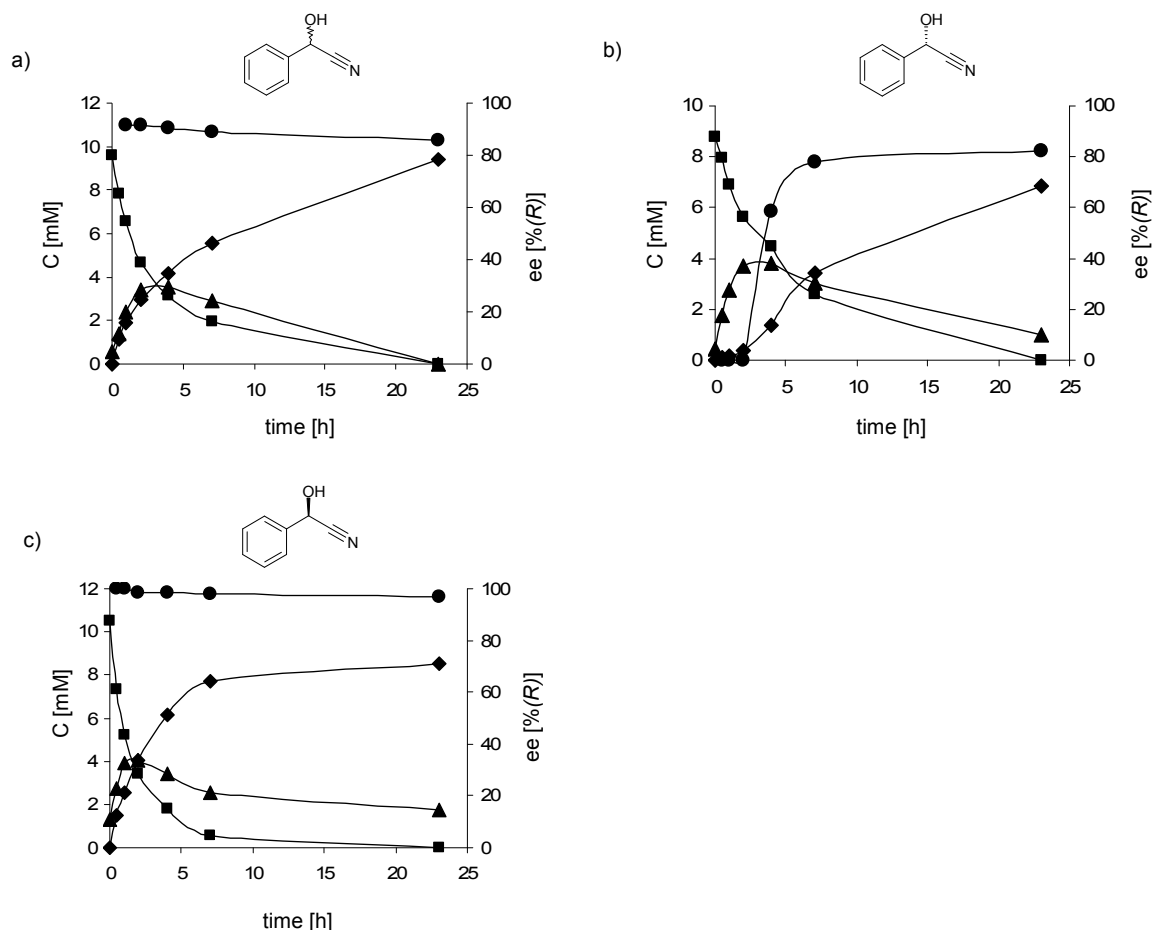


Figure 6. Hydrolysis of (*R,S*)- and (*R*)-**3** (10 mM) by HnNLase (0.29 mg protein (0.58 mg for (*R,S*)-experiment)) at pH 5.5 and 5 °C. Benzaldehyde (▲), mandelic acid (◆), **3** (■), ee (*R*)-mandelic acid (●).

(*R,S*)-**3** was hydrolyzed with a high initial rate (Figure 6a) that progressively slowed down indicating a kinetic resolution type of process. As long as there was enough of (*R*)-enantiomer in the reaction, the enzyme worked at high rate. When the concentration of the latter dropped, the (*S*)-enantiomer, even though not favored by the enzyme, started to be converted at a modest rate. Despite the low pH, a high (3.5 mM) concentration of benzaldehyde was found in the reaction samples due to

spontaneous dehydrocyanation of the nitrile. The reverse reaction led to formation of (*R,S*)-**3**. Consequently, over 20 h reaction time all of (*R,S*)-**3** could be converted into 85% enantiomerically pure acid, only slightly less than the initial ee of 95% (*R*).

The hydrolysis of (*S*)-**3** (Figure 6b) presents further evidence for the strong (*R*)-enantioselectivity of HnNLase. During the reaction mainly the (*R*)-enantiomer, formed from *in situ* racemization of the (*S*)-enantiomer, was converted into (*R*)-mandelic acid. After 2 h, when more (*R*)-**3** started to be available via racemization, the product ee(*R*) increased rapidly. Hence, the enzyme is then mainly converting (*R*)-**3**. When the reaction reached completion, the ee of the (*R*)-mandelic acid was 82%. In the hydrolysis of (*R*)-**3**, in contrast, the high initial rate and the – initially – absolute enantiomeric purity (> 99% (*R*)) proved the high (*R*)-selectivity of the NLase (Figure 6c) and showed a dynamic kinetic resolution type of reaction. After < 10 h all **3** was converted into (*R*)-mandelic acid with 97% ee and leaving some benzaldehyde (about 2 mM).

Nitrile **1**, which is sterically similar to **3**, but bearing a different α -substituent, was hydrolyzed at pH 7 with an initial rate of $0.05 \mu\text{mol mg}^{-1} \text{min}^{-1}$ (Table 1). Also in this case, (*R*)-phenylpropionic acid was formed with 88% initial ee (Figure 7).

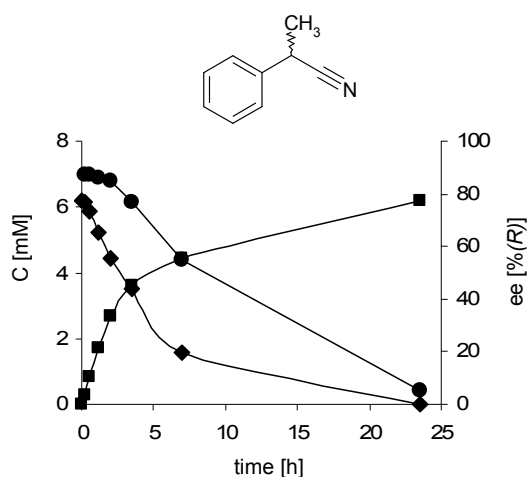


Figure 7. Hydrolysis of (*R,S*)-**1** (10 mM) with HnNLase (0.58 mg protein) at pH 7 and 25 °C. (*R,S*)-2-phenylpropionitrile (◆), 2-phenylpropionic acid (■), ee (*R*)-phenylpropionic acid (●).

According to the experimental data, HnNLase shows high, but not absolute affinity for (*R*)-**1**. There is three times more (*R*)-enantiomer converted by the NLase than (*S*)-enantiomer. When after < 25 h the reaction completed, ee dropped to 0 as should be expected in a kinetic resolution.

Nitrile **2** was the fastest substrate from the whole spectrum of tested nitriles and was converted 15 times as fast as **1**. Interestingly, **2** could not be converted stoichiometrically into phenylacetic acid. Typically under the reaction conditions, about 30% acid was missing when the reaction reached the completion (Figure 8).

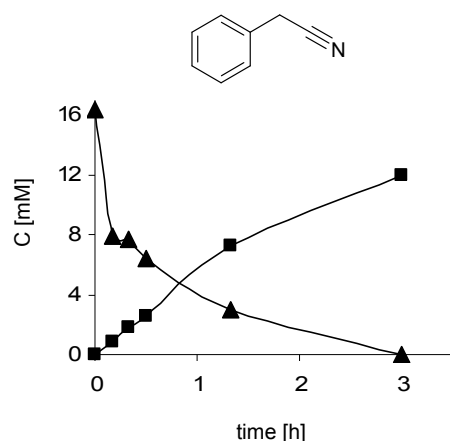


Figure 8. Hydrolysis of **2** (15 mM) with HnNLase (0.12 mg protein) at pH 7 and 25 °C. Phenylacetonitrile (▲), phenylacetic acid (■).

The mass disbalance can possibly be attributed to future metabolism of **2** or its acid by the bacterial cells. In an experiment with washed cells, **2** was anaerobically incubated in phosphate buffer pH 7 (Figure 9). In a separate experiment, phenylacetic acid was incubated with the washed cells in oxygen-free atmosphere in the presence and absence of ammonia.

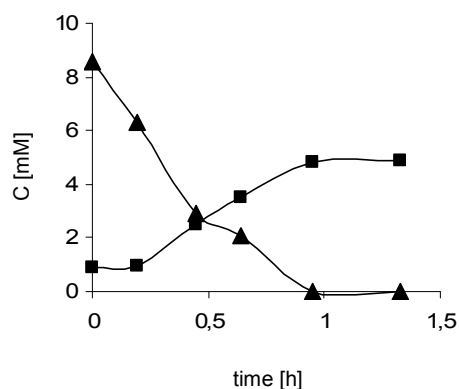


Figure 9. Anaerobic incubation of **2** (10 mM) with HnNLase cells (0.12 mg protein) in phosphate buffer pH 7 at 25 °C. Phenylacetonitrile (▲), phenylacetic acid (■).

The deficit of phenylacetic acid accumulation by the washed cells indicates rapid utilization of the acid. While this would be easy to explain for a growing culture, the

reason for any phenylacetic acid deficit with washed cells is not clear. It would seem that the washed cells could have oxidized phenylacetic acid but the same deficit was apparent in the absence of oxygen (Figure 9). In contrast, no consumption of phenylacetic acid was found upon its incubation with washed cells, in the absence of oxygen. Perhaps, the cells might retain some of the acid formed during the intracellular hydrolysis of **2**. While **2** is clearly xenobiotic, phenylacetic acid is a natural compound with plant hormone activity which occurrence is related to phenylalanine metabolism. Many bacteria can utilize this compound by activation with CoA followed by ring hydroxylation.⁸ In any case the exact mechanism of phenylacetic acid utilization in alkaliphilic *Halomonas* strain needs further detailed investigation not included in this Chapter.⁶

Benzonitrile (**5**) showed only marginal activity but a similar substrate, 3-cyanopyridine (**6**), proved to be 15 times more reactive than **5**. The effect of the pyridyl ring could be caused by its electron-withdrawing nature or by the much better solubility of **6** in aqueous medium.

Analogous to enantiopure α -hydroxy acids, enantiopure amino acids, e.g. phenylglycine, are important pharmaceutical intermediates. D-phenylglycine is a starting material in the production of semisynthetic penicillins and cephalosporins with the estimated market volume of 45 000 ton $\cdot$ y⁻¹ (in 2000).^{9, 10} Phenylglycine derivatives are also used in the synthesis of antitumor drugs.¹¹ A time-course of the conversion of phenylglycine nitrile (**7**) at pH 8.4 is presented in Figure 10.

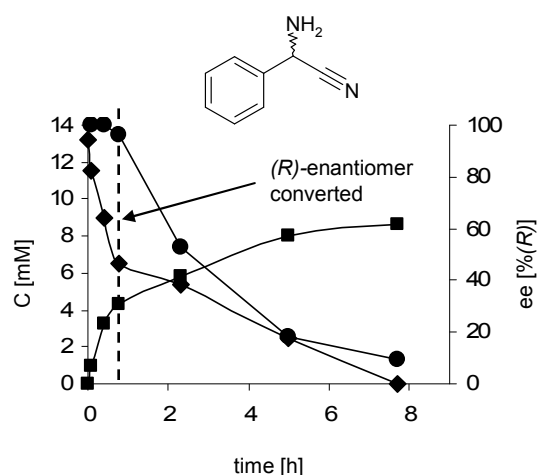


Figure 10. Hydrolysis of (*R,S*)-phenylglycinonitrile (10 mM) with HnNLase (0.58 mg protein) at pH 8.4 and 25 °C. 2-phenylglycinonitrile (◆), phenylglycine (■), ee (*R*)-phenylglycine (●).

The reaction was completed after 7 h and the fastest reaction progress was observed in the first 45 min of the reaction. After the first 45 min virtually only the (*R*)-enantiomer was converted (ee(*R*) 96%), showing a kinetic resolution type of reaction. Then, the reaction continued with hydrolysis of the (*S*)-enantiomer into the corresponding (*S*)-acid. The mass imbalance observed in this reaction can be explained by fast and spontaneous decomposition of **7** to the corresponding benzaldehyde, HCN and ammonia.¹² Unfortunately, none of these decomposition products could be detected by the analytical method used. It would seem, in conclusion, that enantioselective hydrolysis of **7** into (*R*)-phenylglycine in the presence of HnNLase is feasible in principle.

2.4. Dynamic kinetic resolution of (*R,S*)-mandelonitrile

In the previous paragraph, where the reaction kinetics were studied, we found that the stability of the investigated nitriles is of paramount importance. In the conversions of **3**, a low temperature and low pH were selected (pH 5.5) to slow down the chemical decomposition and racemization. On the other hand, the spontaneous racemization could be usefully employed in a chemo-enzymatic dynamic kinetic resolution, to convert (*R,S*)-**3** into enantiomerically pure (*R*)-mandelic acid (see Figure 11). A similar process is industrially applied by BASF¹³ and Mitsubishi¹⁴.

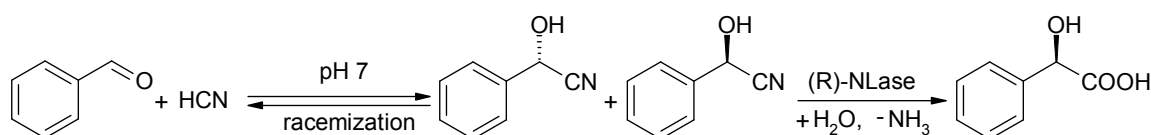


Figure 11. One pot chemo-enzymatic dynamic kinetic resolution process for the synthesis of (*R*)-mandelic acid.

A rapid racemization, in which the nitrile is racemized *via* the (de)hydrocyanation pathway, is obviously required here. This could be achieved, using buffers at higher pH values (preferably pH > 7) and temperatures starting from RT. As mentioned in the previous paragraph, such a process is commercially applied on industrial scale in the chemo-enzymatic synthesis of (*R*)-mandelic acid by BASF and Mitsubishi. Figure

12 shows the differences in the chemical decomposition rates of (*R,S*)-**3** at 25 °C and pH 7.0 (Figure 12a), with comparison to (*S*)-**3** at 5 °C and pH 5.5 (Figure 12b).

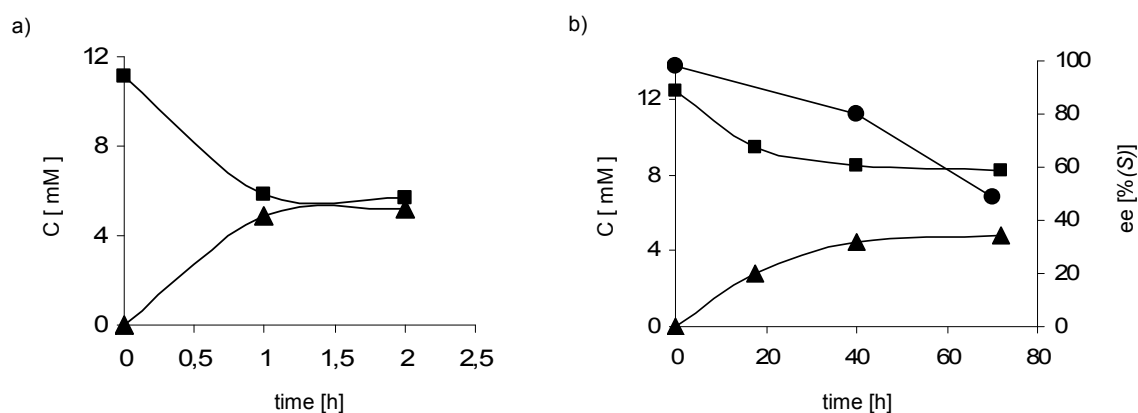


Figure 12. The comparison of the decomposition rates of mandelonitrile at pH 7 and 25 °C (a) and pH 5.5 and 5 °C (b). **3** (■), benzaldehyde (▲) *ee* (*S*)-**3** (●).

In the incubation at pH 7.0 and 25 °C, the decomposition equilibrium sets at about 55% of decomposed **3** (Figure 12a), after less than 1h already, compared with only 25% of decomposed (*S*)-**3** after 20 h at pH 5.5 and 5 °C (Figure 12b).

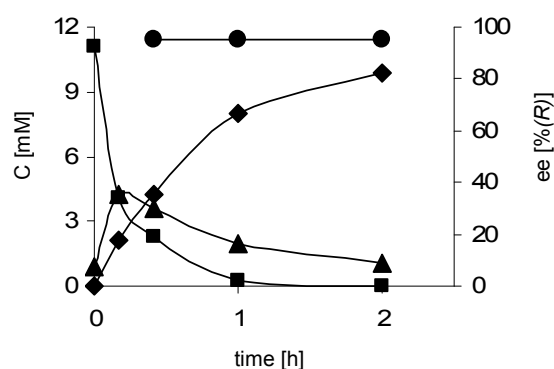


Figure 13. One pot, chemo-enzymatic, dynamic kinetic resolution synthesis of (*R*)-mandelic acid using HnNLase resting cells at pH 7 and 25 °C. (*R,S*)-**3** (■), mandelic acid (◆), benzaldehyde (▲), *ee* (*R*)-mandelic acid (●).

Accordingly, a test reaction with 10 mM (*R,S*)-**3**, showed the system to be effective (Figure 13). The fast racemization assured constant supply of the desired (*R*)-enantiomer of the cyanohydrin, which was then converted by the HnNLase into (*R*)-mandelic acid. Full conversion was reached already after 2h. The enantiopurity of the acid was > 95% (*R*).

2.5. Fed-batch experiments with phenylacetoneitrile, (*R,S*)-mandelonitrile and (*R,S*)-2-phenylglycinonitrile

In the following experiments the opportunities in applying the HnNLase sp. resting cells in conversions with higher concentrations (> 0.1 M) of three different nitriles (**2**, (*R,S*)-**3** and **7**) were studied. In the test, HnNLase cells suspended in phosphate buffer pH 7 were fed with 94 μmol portions of pure **2**. Subsequent portions were added only when the previous one was fully converted into the corresponding phenylacetic acid (Figure 14).

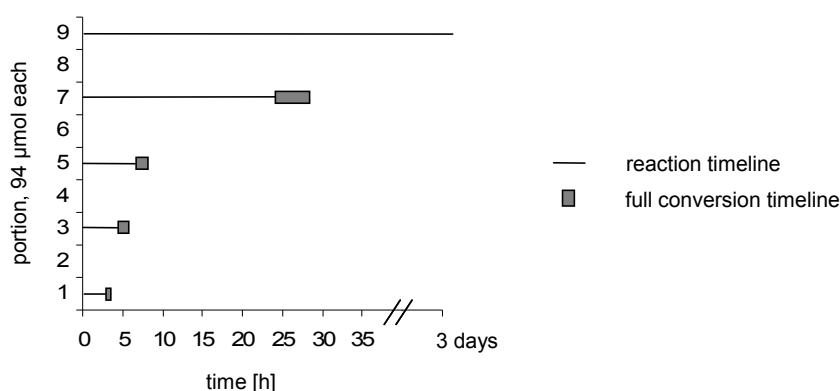


Figure 14. Fed-batch synthesis of phenylacetic acid with HnNLase resting cells at pH 7 and 25 °C.

The first 94 μmol of **2** was converted in less than 3.5 h. With increased concentration of reactants, a gradual decrease of the biocatalyst activity was observed. The last, ninth portion was added in the eighth day of the experiment. The conversion of the last portion was progressing much slower than the earlier ones, and eventually stopped after converting $\sim 40\%$ of the substrate portion. Most likely it was related to the large amount of phenylacetic acid salt in the reaction medium. No pH shift was observed in the experiment. During the test, formation of white crystals occurred. The crystalline substance was identified as phenylacetic acid NH_4 salt, which crystallized out quickly from aqueous solutions due to its very poor solubility in the reaction medium.

Syntheses of the more valuable acids from nitrile racemates with a chiral α -carbon, e.g. (*R,S*)-**3** and **7**, at high concentrations were also investigated. The experiments with **7** showed that the resting cells were also able to fully convert at least 220 μmol (0.2 M) **7**. The first 112 μmol portion was almost fully converted after 24 h.

Unfortunately, a fed-batch test with (*R,S*)-**3** totally failed. This could be due to the nitrile having a high solubility in the reaction medium that in turn might directly influence the HnNLase cells stability.

2.6. Cell disruption and the stability of HnNLase in the extract

Application of HnNLase as a (co)-immobilized biocatalyst requires at least a cell-free extract. Two different approaches to this latter objective were attempted: rapid decompression, which opens cells by a sudden drop of pressure, and osmotic shock, a technique to lyse cells by a decrease in osmotic pressure. We also investigated the effects of process variables such as an anaerobic environment and stabilizing additives on the yield of active protein.

2.6.1. Rapid decompression

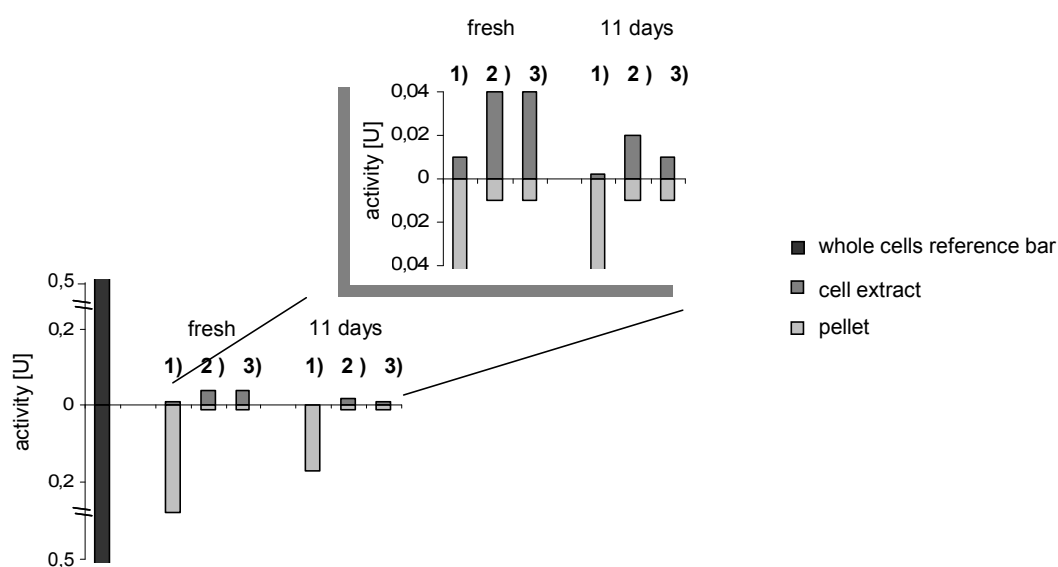
The methodology was applied in three different ways: disruption in buffer at pH 10, buffer pH 10 with addition of glycerol 10% (v/v) and buffer pH 10 with addition of a protease inhibitor (Table 2, entries 1 – 3 respectively). Glycerol is a known protein stabilizer which is generally applied in concentrations not exceeding 40% (v/v)¹⁵ (10% (v/v) in our test) and works by changing the surface tension around the protein while mixed with water.¹⁶ A protease inhibitor was added to inhibit protease(s) that could hydrolyse HnNLase in the cell-free extract.

The cells were disrupted at 2 kbar and resulted in a liquid, non viscous cell-free extract. After separating the soluble protein from the cell debris, the total protein concentration was measured according to standard protein assay protocols.

Table 2. Total protein content in the disrupted solutions from the rapid decompression disruption method experiment.

Sample no.	Total protein [mg]		
	Before disruption	After disruption	
		Extract	Pellet
1)	2.9	0.45	0.87
2)	2.9	2.30	0.41
3)	2.9	1.75	0.62

The protein recovery upon decompression in plain buffer was low, only 45%, for unknown reason. In experiments 2) and 3), however, the cells were opened successfully. Bradford tests revealed that the total protein content in the soluble fraction and the pellet was 2.71 mg (93%) and 2.21 mg (76%) for experiments 2) and 3) respectively.

**Figure 15.** Comparison of HnNLase batch activities in the cell-free samples extracted in the rapid decompression disruption method experiment. Cell sample suspended in sodium carbonate buffer pH 10 (1), sodium carbonate buffer pH 10 + 10% glycerol (2), sodium carbonate buffer pH 10 + protease inhibitor (3). Storage test at 0 °C.

Samples from the extract as well as the pellet were assayed for HnNLase activity at 25 °C (Figure 15). The assay was repeated after 11 days storage at 0 °C. Only a minor part, < 8%, of the original HnNLase batch activity (0.51 U) could be recovered in the extract. Upon decompression in plain buffer (experiment 1) a major part, 60% of the starting activity, was recovered in the pellet. In all cases, the activity was rapidly lost upon storage at 0 °C.

2.6.2. Osmotic shock

The unsatisfactory results from the decompression experiments prompted us to investigate the effects of oxygen on the cell-free HnNLase in the course of our experiments with osmotic shock cell disintegration. Mateo *et al.* have already shown that NLases are highly sensitive to oxygen, which is commonly ascribed to autoxidation of the catalytic cysteine residue.¹⁷

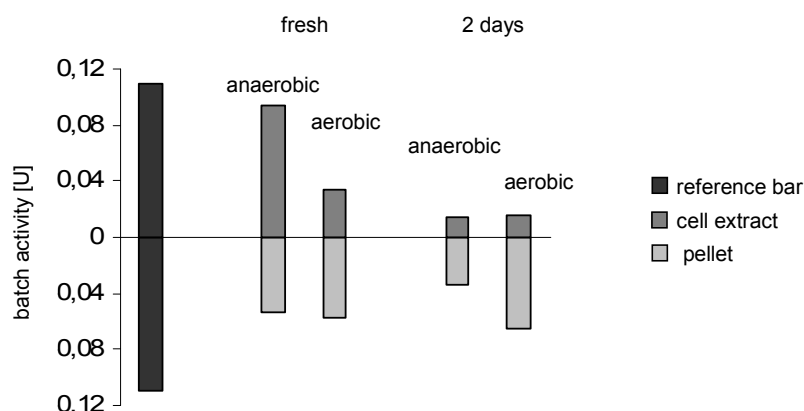


Figure 16. Anaerobic and aerobic Osmotic Shock cell extraction. HnNLase storage stability on ice in 20 mM tris buffer pH 8.

Two sets of experiments comparing oxygen free (anoxic) and aerobic cell disruption were carried out (Figure 16). Upon subjecting the cells to osmotic shock in the absence of oxygen, 64% of the recovered activity (0.09 U) was in the extract and 36% (0.05 U) in the pellet. Repeating the experiment under aerobic conditions afforded a pellet containing approximately the same HnNLase activity (0.06 U) as before, whereas only 0.03 U was recovered in the extract. Apparently, shielding HnNLase from oxygen is of paramount importance. A rapid loss of activity was

observed, as before, upon storage at 0 °C. Summarizing these two experiments it can be concluded that the HnNLase is inherently unstable outside the cell and the cell extract storage under O₂ should be avoided.

3. Conclusions

We have shown that *Halomonas nitrilicus* hydrolyses nitriles via the NLase pathway. The corresponding carboxylic acid is nearly exclusively produced, with negligible amide formation. The HnNLase has a quite relaxed substrate specificity, with a preference for phenylacetone nitrile derivatives. Its potential was demonstrated in a chemoenzymatic dynamic kinetic resolution of mandelonitrile, which afforded (*R*)-mandelic acid of 95% enantiomeric purity. A fed-batch hydrolysis of phenylacetone nitrile resulted in 0.84 M phenylacetic acid.

Extracting HnNLase from the cells is not trivial and is further complicated by its sensitivity for oxygen. From our, somewhat tentative, experiments it would seem that osmotic shock could be developed into an efficient procedure.

4. Materials and methods

Chemicals

Phenylacetone nitrile, phenylacetic acid and benzonitrile were obtained from Merck-Schuchardt; (*R,S*)-mandelic amide 97 % was from Alfa Aesar. 2-Phenylpropionitrile, (*R,S*)-mandelic acid, (*R,S*)-mandelonitrile tech, phenylglycine nitrile hydrochloride +95 % tech., (*R*)-D-(−)-phenylglycinamide 99 %, (*R*)-D-(−)-phenylglycine 98 % and (*S*)-L-(+)-phenylglycine 99 % were from Acros Organics; (*R,S*)-2-phenylpropionic acid was obtained from Janssen Chimica. Benzoic acid, acrylic amide +99 % and 3-cyanopyridine 98% were from Aldrich. Veratrol +99 %, (*R*)-D-(−)- and (*S*)-L-(+)-mandelic acid, nicotinamide +99.5 %, nicotinic acid +99.5 %, acrylonitrile +99.5 % and acrylic acid +99 % were purchased from Fluka. Phenylacetamide was from the laboratory stock of in-house synthesized chemicals. Protease inhibitor (Complete

mini, EDTA-free) was from Roche. DNase (Deoxyribonuclease from Bovine pancreas), Bradford reagent and Lysozyme were purchased from Sigma.

(S)- and (R)-mandelonitrile were synthesized from benzaldehyde and HCN as previously described in one of our previous papers.¹⁸ (R,S)-2-Phenylpropionic amide was synthesized enzymatically by hydrolysis of 2-phenylpropionitrile (1.31 mg, 10 mM) in the presence of 3.9 mg nitrile hydratase CLEA¹⁹ in 1.0 mL sodium carbonate buffer pH 10. When all the nitrile was converted into the corresponding amide, the solution was spun down, and the solution containing 10 mM (R,S)-2-phenylpropioamide was stored at 4 °C.

Instrumental analysis

Nitrile, amide and carboxylic acid concentrations during the reaction were determined by HPLC. The conversions of (R,S)-mandelonitrile, phenylacetonitrile, benzonitrile and (R,S)-2-phenylpropionitrile were monitored by reversed phase HPLC on a 4.6 x 50 mm Merck Chromolith SpeedROD RP-18e column in an Alliance Waters 2695 Separation Module equipped with a Waters 2487 Dual λ UV Absorbance Detector at 215 nm. Mobile phase H₂O-ACN (90:10, v/v) containing 0.1% (v/v) TFA (1 mL min⁻¹). The enantiomeric purity of the hydrolysis products of (R,S)-mandelonitrile and 2-phenylpropionitrile were determined by straight-phase chiral HPLC on a Chiralcel 4.6 x 250 mm Chiralpak AD-H column, a Waters M 515 pump and a Waters M 486 Tunable Absorbance Detector at 215 nm. Eluant hexane-isopropyl alcohol 80:20 ((R,S)-3) and 90:10 (2) + 0.1% (v/v) TFA (0.5 mL·min⁻¹).

The conversion of phenylglycinonitrile and the enantiopurities of the corresponding acids were determined using the same HPLC module as above. Separation was obtained using a Daicel Crownpak CR(+) column and H₂O + 0.1% (v/v) HClO₄ as eluent (0.5 mL·min⁻¹).

The conversions of acrylonitrile and 3-cyanopyridine were monitored by reversed-phase HPLC on three 4.6 x 50 mm Merck ChromolithTM SpeedROD RP-18e columns in series for acrylonitrile and a single column for 3-cyanopyridine, a Waters 590 programmable pump and a Shimadzu SPD-10A VP UV-VIS detector at 210 nm. Eluant water containing 0.1% (v/v) TFA (acrylonitrile) or acetic acid (3-cyanopyridine) the kinetic assay experiments and conversion experiments were carried out on Q.Instruments ThermoTWISTER comfort shaker.

Protein content measurements

For the measurements of the total protein content in the whole cells and the protein extract, Lowry/Bradford and Bradford methods were used respectively.^{20, 21} The samples were measured on Shimadzu UV-240IPC spectrophotometer.

Culture of *H. nitrilicus* sp. nov. strain ANL- α CH3

A mineral medium based on sodium carbonate buffer at pH 10 and 0.6 M total Na⁺ was used for the growth of the enriched strain. The buffer was composed of [g·L⁻¹]: Na₂CO₃, 22; NaHCO₃, 8; NaCl, 6; K₂HPO₄, 0.5. After sterilization, the medium was supplemented with 2 mL 1:1 mixture of trace metals solution²² and 20% MgSO₄·7 H₂O, 10 mL 2 M Na₂S₂O₃·5 H₂O, 10 mL 2 M H₃CCOONa and 2 mL 50 mg·L⁻¹ filter-sterilized vitamin B₁₂. The growth was carried out in 2 L Erlenmeyer's flask in 200 mL growth medium sodium carbonate buffer at pH 10. The medium was inoculated with 200 μ L enriched *Halomonas nitrilicus* culture from -80 °C glycerol stock. The substrate (*R,S*)-2-phenylpropionitrile was added in 1 mM portions. The growth of the pure culture was carried out in a 2" orbit rotary shaker (Innova 44, New Brunswick Scientific) at 30 °C and 150 rpm. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) in a Biowave II spectrophotometer (WPA). The degradation of the nitrile was monitored by HPLC (as described in the instrumentation part for 2-phenylpropionitrile). When the growth reached the stationary phase, the bacterial material was centrifuged at 10k rpm for 15 min. The cells were resuspended in the growth medium pH 10 at 5.8 mg protein·mL⁻¹ cell density, distributed in equal portions in 1.5 mL eppendorfs, and stored on ice. The protein content and activity of the enzyme were measured as described in the concerning paragraphs.

pH Profile of HnNLase

The pH profile was measured in two buffers, containing 0.6 M Na⁺: pH 5 – 8 0.1 M HEPES/NaCl; pH 8.5 - 11.5 in NaHCO₃/Na₂CO₃/NaCl buffer. Final cell density was 0.1 mg protein·mL⁻¹, final volume; 2 mL in 2 mL eppendorf tubes at 30 °C. The final pH was also measured and the values in the graph represent final pH values. This is important to stress, because most of the publications on "alkaliphiles" do not report final pH values and use weak buffers, therefore the data may be unreliable. Reaction

rates (ammonia formation) were measured at regular intervals after 10 seconds centrifugation of 0.1 mL samples at 13k rpm. To determine the concentration of the hydrolysis products, the ammonia content was measured using the phenol-hypochlorite method.²³

Amidase activity in HnNLase

Mandelic amide: a 1.5 mL eppendorf was charged with 870 μ L 20 mM citrate buffer pH 5.5, 30 μ L (0.78 mg protein) cell suspension, 2 μ L internal standard (2 M Veratrol solution in MeOH); finally 100 μ L (*R,S*)-mandelic amide (0.1 M solution in citrate buffer) was added to start the reaction. The reaction was incubated on a thermo-shaker at 25 °C. 10 μ L Reaction samples were taken periodically, diluted in HCl acidified mobile phase, spun down to separate the cells, and analyzed by HPLC.

Phenylpropionic amide: a 1.5 mL eppendorf was charged with 850 μ L 10 mM sodium carbonate buffer pH 10, 100 μ L (*R,S*)-phenylpropionic amide (0.1 M solution in the bicarbonate meeting) to make the final volume of 0.95 mL and then 50 μ L (0.29 mg total protein) cell suspension to start the test. The reaction was incubated on a thermo-shaker at 25 °C. The samples were analyzed as described in the previous paragraph.

Activity assays and tests for substrate specificity

(*R,S*)-, (*S*)- and (*R*)-mandelonitrile: 1.5 mL eppendorfs with 100 μ L cells suspension (0.58 mg) (for (*R*)- and (*S*)- experiment), 50 μ L (0.29 mg protein) ((*R,S*)-mandelonitrile experiment at 5 °C) and 20 μ L (0.12 mg protein) (for ((*R,S*)-mandelonitrile experiment at 25 °C), were spun down two times in 20 mM citrate buffer pH 5.5 to wash the cells from the growth media buffer. Then, the resting cells were resuspended in the citrate buffer to 1 mL final volume (after addition of all reactants). 2 μ L internal standard (2 M Veratrol solution in MeOH) and finally 5 μ L mandelonitrile (2M solution in MeOH) was added to start the reaction. The reaction was incubated on a thermo-shaker at given temperatures. 10 μ L reaction samples were taken periodically, diluted in HCl acidified mobile phase, and after centrifugation to separate the cells, analyzed by HPLC. The samples for enantiomeric ratio measurements, were first extracted from the aqueous phase to ethylacetate, diluted

with the mobile phase, dried over MgSO_4 and then measured on the Chiralpak AD-H column.

Phenylacetonitrile, 2-phenylpropionitrile and benzonitrile: 100 μL cell suspension (0.58 mg) and 20 μL (0.12 mg) for phenylacetonitrile were washed and diluted as described above, but with 0.1 M phosphate buffer pH 7. Internal standard and the substrates were added from 2 M stock solutions in MeOH. The reactions were carried out on the thermo-shaker at 25 $^\circ\text{C}$. HPLC samples were prepared and analyzed as described above. The samples for ee measurements of 2-phenylpropionitrile hydrolysis products were prepared in the analogous way to mandelonitrile samples.

(*R,S*)-phenylglycinonitrile, acrylonitrile and 3-cyanopyridine: 100 μL cell suspensions (0.58 mg) were washed and diluted as described above. No internal standard was used in these reactions. The substrates were added from 0.1 and 0.5 M stock solutions in phosphate buffer and 1 M stock solution in MeOH respectively. The reactions were carried out on the thermo-shaker at 25 $^\circ\text{C}$. HPLC samples were prepared and analyzed as described above.

Dynamic kinetic resolution experiment with (*R,S*)-mandelonitrile

200 μL (1.16 mg protein) *H. nitrilicus* resting cells suspension were placed in a 1.5 mL eppendorf and spun down two times in 0.1 mM phosphate buffer pH 7 to remove the sodium carbonate buffer pH 10 from the cells. Then, the resting cells were resuspended in the phosphate buffer to 1 mL final volume (after addition of all reactants). 2 μL internal standard (2 M Veratrol solution in MeOH) and finally 5 μL (*R,S*)-mandelonitrile (2M solution in MeOH) was added to start the reaction. The reaction was incubated on a thermo-shaker at 25 $^\circ\text{C}$. Samples were measured as described for experiments with the same substrate.

Fed-batch experiments

Phenylacetonitrile, (*R,S*)-mandelonitrile and (*R,S*)-2-phenylglycinonitrile: 150 μL *H. nitrilicus* cell suspension (0.87 mg) in a 1.5 mL eppendorf was spun down two times in 0.1M phosphate buffer pH 7.0. Then, the resting cells were resuspended in the phosphate buffer to 1 mL final volume. 12 μL pure phenylacetonitrile, 27 μL (*R,S*)-mandelonitrile and 16 μL (*R,S*)-2-phenylglycinonitrile were added (in separate experiments) to start the reaction. The next substrate portions were added only after the previous one was fully converted. The reaction was carried out on the thermo-

shaker at 25 °C. Reaction samples were taken periodically, diluted in HCl acidified mobile phase, and after centrifugation to separate the cells, analyzed by HPLC.

Enzyme extraction experiments

Rapid decompression disruption method: The cell samples containing 2.9 mg protein were diluted 10 times with sodium carbonate buffer pH 10.1 to give the final 5 mL. Sample 2) contained additionally 10% (v/v) glycerol and 3) a small portion of a protease inhibitor. The disruptions were carried out (IKS Lab Equipment Constant Cell Disruption System) at 2k bar and at RT. The obtained suspensions of the cell-free proteins, crushed cell walls and insoluble proteins were spun down at 30k rpm at 4 °C for 0.5 h (Sorvall RC-5B refrigerated superspeed centrifuge). The supernatant with the free enzyme, and other soluble proteins was separated from the insoluble proteins and the cell debris. The pellet was resuspended in 0.5 mL sodium carbonate buffer pH 10. The protein content in all samples was measured according to the Bradford procedure. The batch activity was assayed in the following way: the enzyme sample was incubated with (*R,S*)-mandelonitrile (10 mM) in 20 mM citrate buffer pH 5.5 in the presence of 4 mM internal standard at 25 °C. Samples were diluted and filtered over Microcon YM-10 centrifugal filters and analyzed as described in the instrumentation part.

Osmotic shock method: Two sets of enzyme extraction experiments were simultaneously carried out. First, under the anaerobic conditions (anaerobic tent) at 30 °C, and second, under aerobic conditions at 22 °C. HnNLase whole cell samples (0.58 mg protein) in 1.5 mL eppendorfs, were centrifuged for 0.5 min at 13.4k rpm. For the cell opening experiments, the supernatant was discarded and the cells were resuspended in 1.0 mL 20 mM tris buffer pH 8.0 containing 1 mg·mL⁻¹ lysozyme and a small portion of DNase. The osmotic shock experiment was carried out on a magnetic stirrer for 1 h. During this time, the samples were kept on ice. The crude enzyme extracts were centrifuged for 5 min at 13.4k rpm. Cell-free, soluble proteins were separated from the cell debris. Batch activities (as described in the upper paragraph) of both the soluble and the insoluble parts were assayed under anaerobic and aerobic conditions at the local temperatures. The activities were measured again after two days of storing the samples on ice.

5. Reference list

1. Sorokin, D. Y.; van Pelt, S.; Tourova, T. P.; Takaichi, S.; Muyzer, G. *Microbiology-Sgm* **2007**, *153*, 1157-1164.
2. Sorokin, D. Y.; Pelt, S. v.; Tourova, T. P.; Muyzer, G. *Appl. Environ. Microbiol.* **2007**, *73*, 5574-5579.
3. Foti, M.; Sorokin, D. Y.; Lomans, B.; Mussman, M.; Zacharova, E. E.; Pimenov, N. V.; Kuenen, J. G.; Muyzer, G. *Appl. Environ. Microbiol.* **2007**, *73*, 2093-2100.
4. Sorokin, D. Y.; Zhilina, T. N.; Lysenko, A. M.; Tourova, T. P.; Spiridonova, E. M. *Extremophiles* **2006**, *10*, 213-220.
5. Quillaguaman, J.; Hashim, S.; Bento, F.; Mattiasson, B.; Hatti-Kaul, R. *J. Appl. Microbiol.* **2005**, *99*, 151-157.
6. Chmura, A.; Shapovalova, A. A.; van Pelt, S.; van Rantwijk, F.; Tourova, T. P.; Muyzer, G.; Sorokin, D. Y. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 371-378.
7. Alva, V. A.; Peyton, B. M. *Environ. Sci. Technol.* **2003**, *37*, 4397-4402.
8. Navarro-Llorens, J. M.; Drzyzga, O.; Perera, J. *Arch. Microbiol.* **2008**, *190*, 89-100.
9. Mueller, U.; Huebner, S. In *Microbial Production of L-Amino Acids*; Faurie, R.; Thommel, J. Eds. Economic Aspects of Amino Acids Production. Springer: 2003; pp. 137-171.
10. Janssen, M. *Properties of Immobilised Penicillin G Acylase in β -Lactam Antibiotic Sythesis*; Technische Universiteit Delft, 2006.
11. Ravichandran, S.; Dattagupta, J. K.; Chakrabarti, Ch. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1998**, *54*, 499-501.
12. Rustler, S.; Müller, A.; Windeisen, V.; Chmura, A.; Fernandes, B. C. M.; Kiziak, C.; Stolz, A. *Enzyme Microb. Technol.* **2007**, *40*, 598-606.
13. Breuer, M.; Ditrich, K.; Habicher, T.; Hauer, B.; Kessler, M.; Sturmer, R.; Zelinski, T. *Angew. Chem., Int. Ed.* **2004**, *43*, 788-824.
14. Yamaguchi, Y., Ushigome, M., and Kato, T. Process for producing alpha-hydroxy acid or a-hydroxyamide by microorganism. 08/745918 [EP-A-0610048]. 1998. 11-8-1996.
15. Gekko, K.; Timasheff, S. N. *Biochemistry* **1981**, *20*, 4677-4686.
16. Ruan, K.; Xu, Ch.; Li, T.; Li, J.; Lange, R.; Balny, C. *Eur. J. Biochem.* **2003**, *270*, 1654-1661.
17. Mateo, C.; Fernandes, B.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *J. Mol. Catal. B: Enzym.* **2006**, *38*, 154-157.

18. Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1655-1661.
19. van Pelt, S.; Quignard, S.; Kubáč, D.; Sorokin, D. Y.; van Rantwijk, F.; Sheldon, R. A. *Green Chem.* **2008**, *10*, 395-400.
20. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.
21. Gerhardt P.; Murray R.G.E.; Wood W.A.; Krieg N.R. In *Methods for General and Molecular Bacteriology*; Chemical Analysis. American Society for Microbiology: 1994; pp. 554.
22. Pfenning, N.; Lippert, K. D. *Arch. Microbiol.* **1966**, *55*, 245-256.
23. Weatherburn, W. M. *Anal. Chem.* **1967**, *39*, 971-974.

- I Surfactant Catalyzed Hydrocyanations**
- II Asymmetric, One-Pot, Chemo- Enzymatic
Synthesis of Protected Cyanohydrins**

8

1. Introduction

Biotechnology applies surfactants as free-enzyme stabilizers or as stabilizers in enzyme immobilization procedures.^{1, 2} They can also be used as emulsifiers in biphasic (enzymatic) reactions. In the latter case the emulsifier will assure better interfacial mass transfer and better dispersion of the immobilized enzyme in the reaction medium. Surfactants are the primary molecular constituents of micelles. Surfactants undergo self assembly into micelles due to physical forces in order to lower the solution free energy.³ Figure 1 shows the visualization of normal and reverse micelles. The picture of the latter however, is idealized and such packing possibly cannot be obtained without generating energetically unfavorable vacuum cores in totally water-free hydrophobic systems.^{3, 4}

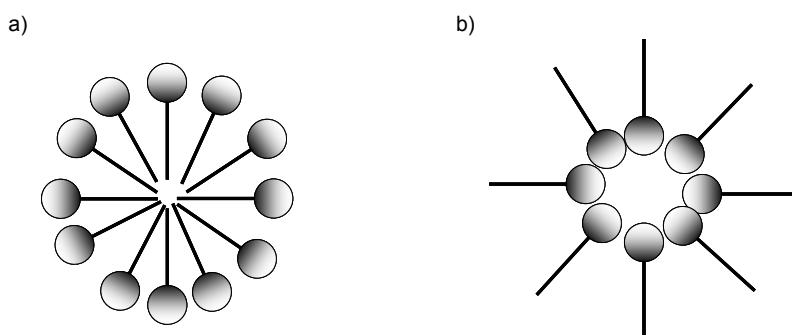


Figure 1. The structures of: spherical micelle (a) and reverse micelle (b).

There is much less known in terms of physical data about catalysis by reverse micelles compared with normal micelles. Normally, reverse micelles contain hydrating water molecules around the surfactant head-groups which fill the space between the heads, forming reverse microemulsions that can work as nanoreactors (sometimes micellar solutions are also viewed as a microheterogeneous systems).³ A literature search has revealed many examples of surfactant micelles being catalytically active. Normal micelles catalyzed e.g. Diels-Alder⁵ or epoxidation reactions⁶, reverse micellar systems catalyzed e.g. phosphorylation of pyrimidinophanes⁷, cyanation of alkyl halides and nucleophilic aromatic substitution.⁸ Micellar catalysis is industrially applied, for example, in emulsion polymerization.⁹ There is not much known, in contrast, about micelle catalyzed hydrocyanation reactions. One example of such a

reaction was found in the work by Jackson *et al.*¹⁰ They report that sodium diisooctylsulfosuccinate influenced the hydrocyanation rate of 3-phenoxybenzaldehyde in toluene. In this Chapter we will show that surfactants (nonionic and ionic) can exhibit catalytic properties in hydrocyanation reactions. It will be shown that they can effectively catalyze formation of cyanohydrins from various aldehydes or ketones with HCN in an anhydrous organic solvent. In the second part of the Chapter, the application of the most productive surfactants as hydrocyanation and racemization catalyst in the one-pot, asymmetric, chemo-enzymatic synthesis of (S)-mandelonitrile (**2**) acetate starting with benzaldehyde (**1**) will be described.

2. Results and discussion

2.1. Enzymatic hydrocyanation of benzaldehyde in a biphasic system. Sodium dodecyl sulfate as emulsifying agent

In an aqueous-organic biphasic reaction system, the biocatalyst tends to stick to the walls of the reaction vessel. Adding sodium dodecyl sulfate (SDS) solved this latter problem at buffer contents > 2%. At lower buffer concentrations, however, the reaction rate and ee suffered (Figure 2).

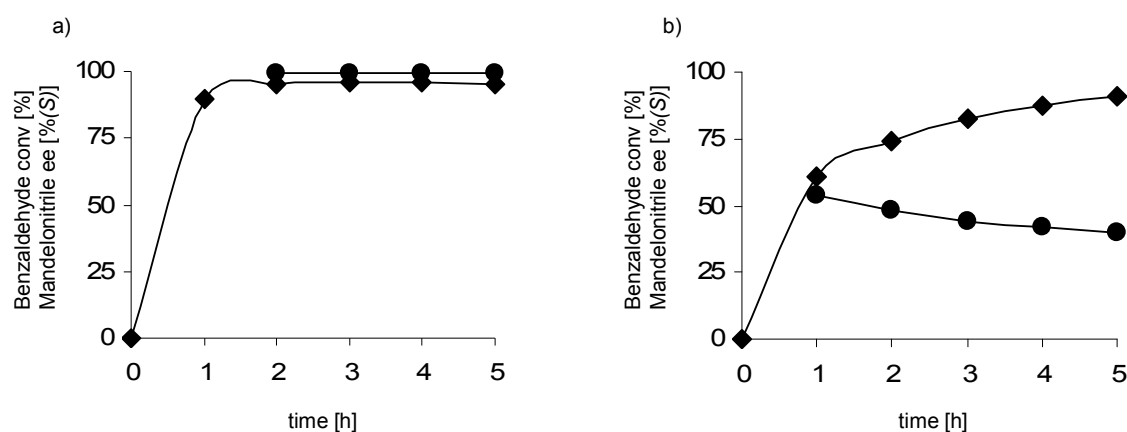


Figure 2. Hydrocyanation of **1** (10 mM) with HCN (70 mM) and 0.10 mg (7.8 U) *M. esculenta* (S)-hydroxynitrile lyase in the presence of biphasic, microaqueous system (98% DIPE and 2% 20 mM citrate buffer pH 5.5) without SDS (a) and with SDS (5 mg) (b). **1** conversion (◆), ee (S)-**2** (●).

The hydrocyanation catalyzed by (*S*)-hydroxynitrile lyase with no SDS added (Figure 2a), was complete in 1 h and ee's of + 99% (*S*) were measured. The same reaction but with SDS added (Figure 2b), in contrast, progressed significantly slower and the product ee after 1 h was only 55% (*S*). The lower enzymatic reaction rate in the latter case could be caused by removal of water by SDS, as a water deficit will reduce the enzyme's performance but will not suppress it.¹¹ The low product ee, on the other hand, indicates competition by a non-selective hydrocyanation reaction. This led us to tentatively conclude that SDS in a micro-aqueous system accelerates the non-enzymatic hydrocyanation.

I. Surfactant mediated hydrocyanation

2.2. Influence of buffer contents on SDS mediated hydrocyanation of benzaldehyde

We investigated the effects of the buffer concentration on the non-enzymatic (SDS-mediated) hydrocyanation of **1**. In the following experiments, the amount of discrete aqueous phase (citrate buffer pH 5.5) in diisopropyl ether (DIPE) was varied from 0 (buffer saturated DIPE) to 2% (Table 1).

Table 1. The influence of the aqueous phase content on SDS catalyzed hydrocyanation of **1**.

Buffer phase [%]	SDS [mg]	Conversion of 1 [%]
0 (saturated)	0	4
	5.3	96
0.2	0	4
	4.8	92
2	0	11
	5.4	18

Reaction conditions: buffer pH 5.5, DIPE, **1** (10 mM), HCN (70 mM), 25 °C.
The conversions were measured after 5 h.

The rate of the background reaction in the absence of any catalyst was low and increased with the water content. When SDS was added, in contrast, the

hydrocyanation was complete after 5 h when the buffer phase was $\leq 0.2\%$, but strongly decreased when more buffer phase was added. Further experiments in which reactants and the reaction medium had been dried over Na_2SO_4 or molecular sieves showed a further gain in the hydrocyanation rates (data not presented).

Taking into account the natural behavior of surfactants in water-free solvents, we concluded that SDS forms reverse micelles that catalyze the hydrocyanation. A possible explanation is that “entrapment” of the reagents in the reverse micelle’s core causes an increase in the molecules’ collision frequency.³ Alternatively, the reverse micelle core could provide a stabilizing environment for the transition state of the hydrocyanation reaction. A similar effect is observed in water based hydrocyanation reactions.

2.3. Hydrocyanation of benzaldehyde with other surfactants. Productivity measurements

Tests with two different ionic surfactants (SDS and tetrabutylammonium chloride) and eight nonionic ones (Tween 80, PEG 8000, sucrose monocaprato monolaurate, sorbitan sesquioleate, monostearate and tetrabutylammonium chloride, Triton X 100 and *n*-octyl glucoside) revealed that these were also capable of catalyzing hydrocyanation. The productivities (according to the definition presented in the Materials and methods) were calculated and are presented in Table 2.

Table 2. Productivity of surfactants in hydrocyanation reactions of **1**.

Surfactant	Mol. mass [g mol ⁻¹]	Productivity [g g ⁻¹ min ⁻¹]
Tween 80	n.a.*	$1.7 \cdot 10^{-2}$
Sorbitan sesquioleate	1109.6	$1.3 \cdot 10^{-2}$
Triton X 100	n.a.*	$1.2 \cdot 10^{-3}$
Sucrose monocaprato	496.6	$1.1 \cdot 10^{-3}$
PEG 8000	8000	$1.1 \cdot 10^{-3}$
<i>n</i> -Octyl glucoside	292.4	$9.1 \cdot 10^{-4}$
Sucrose monolaurate	524.6	$6.0 \cdot 10^{-4}$
SDS	288.4	$1.4 \cdot 10^{-4}$
Sorbitan monostearate	430.6	$1.2 \cdot 10^{-4}$
Tetrabutylammonium chloride	277.9	n.a.**

Reaction conditions: **1** (10 mM), HCN (70 mM), 25 °C.

* mixture of surfactants with different chain lengths

** the value not measured due to phase separation problems

Anionic and nonionic surfactants are capable of catalyzing the hydrocyanation reaction in the anhydrous medium. The highest productivities were achieved with Tween 80 and sorbitan sesquioleate ($1.7 \cdot 10^{-2}$ and $1.3 \cdot 10^{-2}$ g g⁻¹ min⁻¹ respectively). These are over ten times more productive than the third most active surfactant (Triton X 100) and 10^2 more active than SDS and were selected for further experiments. There was also a fast reaction observed with tetrabutylammonium chloride, comparable with Tween 80, but tetrabutylammonium chloride formed an immiscible second phase that was complicating the use of the surfactant in the studies.

2.4. Application of surfactants in hydrocyanation of different aldehydes and ketones

Tween 80 and sorbitan sesquioleate were applied in anhydrous (molecular sieve dried) hydrocyanation reactions with five different aldehydes and ketones (Figure 3).

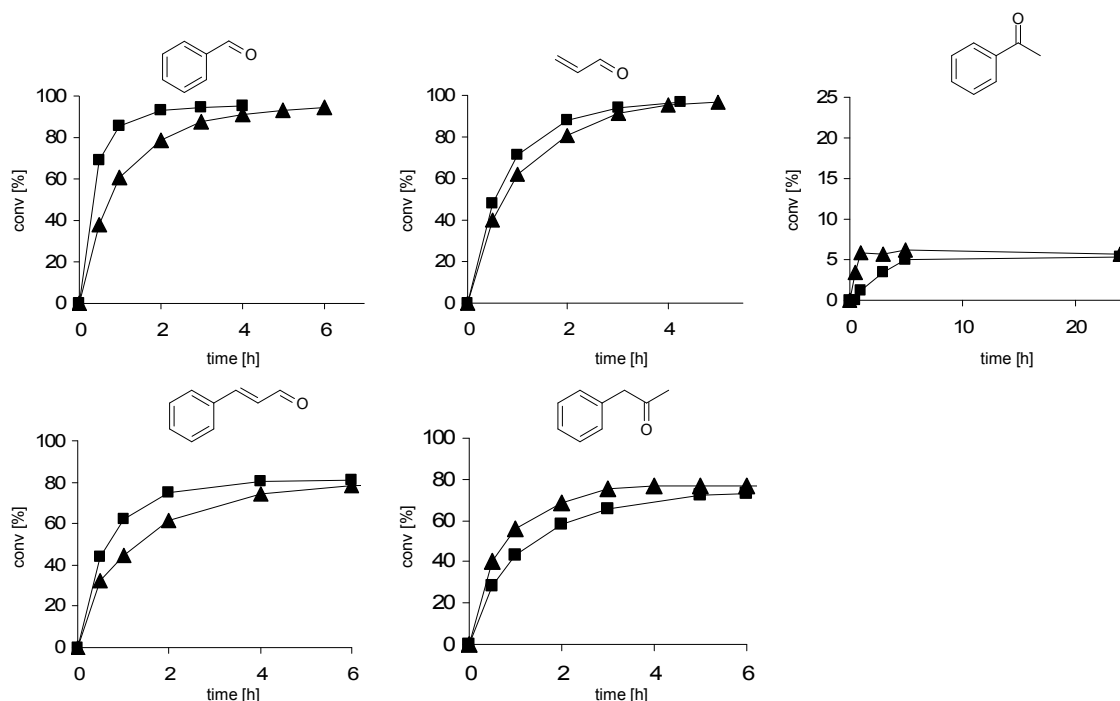


Figure 3. Surfactant catalyzed (50 mg, 1 mg in the reaction with acrolein) hydrocyanation of different aldehydes and ketones (10 mM) with HCN (60 mM) in anhydrous DIPE, Tween 80 (▲), sorbitan sesquioleate (■).

The two selected surfactants were able to catalyze the hydrocyanation of all five aldehydes and ketones until equilibrium had been reached. Only with cinnamic aldehyde the final conversion was somewhat lower than found in otherwise similar experiments described by us earlier, with the same cinnamic aldehyde and HCN concentrations (Chapter 2, Table 1). The reaction rate in the absence of surfactants were negligible. The above set of experiments proved the universal catalytic properties of the selected surfactants in hydrocyanation reactions in anhydrous DIPE. The hydrocyanation of acrolein was significantly faster than that of **1**. The hydrocyanation of acetophenone and 1-phenylpropanone proceeded at a rate comparable with that of cinnamic aldehyde, and all the mentioned reactions were complete in < 4 h. Only a few percent of acetophenone was converted at equilibrium, as would be expected.¹¹

II. Asymmetric, one-pot, chemo-enzymatic synthesis of protected cyanohydrins

A practical application of surfactants in catalysis would be to use them as hydrocyanation and racemization catalysts in an asymmetric one-pot, chemo-enzymatic synthesis of protected (*S*)-cyanohydrins e.g. (*S*)-**2** acetate, where a lipase enantioselectively converts (*S*)-**2** into the corresponding (*S*)-**2** acetate (Figure 4).

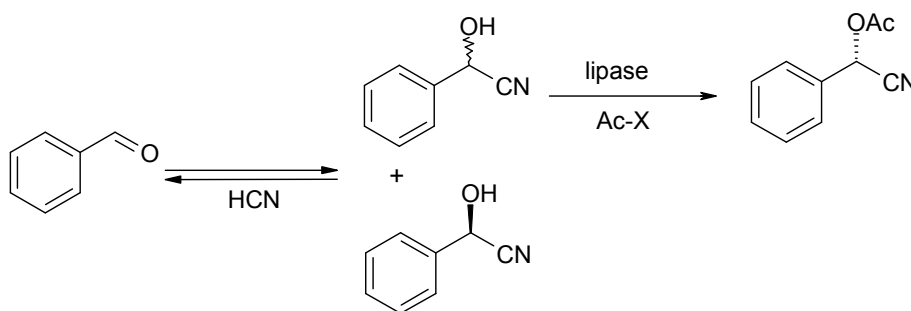


Figure 4. Asymmetric, one-pot chemo-enzymatic synthesis of protected (*S*)-**2** acetate.

Unlike a kinetic resolution, where the maximum yield is 50%, in a one-pot dynamic kinetic resolution (DKR) a 100% yield can theoretically be achieved, because the unconverted enantiomer is simultaneously racemized. A somewhat similar system has been described in which the cyanohydrin is enantioselectively acylated by a lipase while the slow-reacting enantiomer is racemized by a base.^{12,13} The reported drawbacks are the very long reaction times and low yields and enantiopurities.¹⁴ In the following part of the Chapter our efforts to construct and improve the process pictured in Figure 4 will be described. The first, chemical step was catalyzed by the previously selected Tween 80, sorbitan sesquileate and Triton X 100.

2.5. The selection of the enzyme and acetylating agent

The application of lipases in the enantioselective acylation/protection of cyanohydrins is well-established. It was shown that *Candida antartica* lipase B (CALB) or its immobilized version (Novozym 435) can readily work in organic solvents and enantioselectively (with the preference towards *S*-enantiomers) acylate racemic cyanohydrins.¹⁵⁻¹⁷ The choice regarding the acylating agent is an important issue.

Reagents such as vinyl acetate, vinyl butyrate or acetic anhydride give by-products (acetaldehyde and acetic acid respectively) that have a negative influence on CALB and in some cases on the enzyme's enantioselectivity as well. Isopropenyl acetate, on the other hand, is compatible with the lipase and the byproduct is an enol that quickly tautomerizes to give acetone¹⁸⁻²⁰ which is less reactive than acetaldehyde or acetic anhydride. Initial experiments showed that Novozym 435 is able to catalyze the acylation of (*R,S*)-**2** into (*S*)-**2** acetate (99% ee (*S*)) in DIPE as solvent.

2.6. Isopropenyl acetate inhibition effect on the surfactants. The inhibition of Novozym 435 by benzaldehyde and HCN

The tests in which isopropenyl acetate was incubated with the surfactants showed an inhibiting effect of the ester on the hydrocyanation of **1** (Table 3). From the three surfactants sorbitan sesquileate was the most affected by the acetylating agent. In the presence of the ester **1** was converted 7.5 times slower than in the reaction without isopropenyl acetate. Triton X 100 in contrast, lost only 50% of its nominal hydrocyanation rate.

Table 3. The effect of isopropenyl acetate on surfactant catalyzed hydrocyanation of **1**.

	Hydrocyanation [%]			
	Sorbitan sesquileate	Triton X 100	Tween 80	Control reaction
Without isopropenyl acetate	45.0	77.6	66.0	0.0
With isopropenyl acetate	6.0	36.7	11.4	0.4

Reaction conditions: **1** (0.1 M), HCN (0.6 M), isopropenyl acetate (0.3 M), 25 °C. The conversions were measured after 1 h reaction.

Additional tests revealed the effects of the isopropenyl acetate concentration on the rate of hydrocyanation in the presence of Triton X 100 (Figure 5).

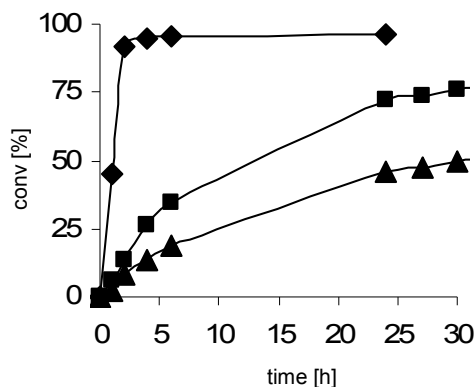


Figure 5. Influence of the isopropenyl acetate concentration on Triton X 100 (20 mg) catalyzed hydrocyanation of **1** (0.1 M) with HCN (0.6 M) in anhydrous DIPE. Control reaction (♦), 300 mM (■), 600 mM (▲).

The hydrocyanation was approximately six times slower when 300 mM isopropenyl acetate was added. Possibly, the catalyst inhibition is caused by the relatively high polarity of isopropenyl acetate that may prevent the assembly of reversed micelles. There was no visible effect of the surfactants and the acylating agent on the CALB catalyzed acylation (data not shown). There was however, a very significant effect of **1** and HCN on the catalytic activity of Novozym 435 (Figure 6).

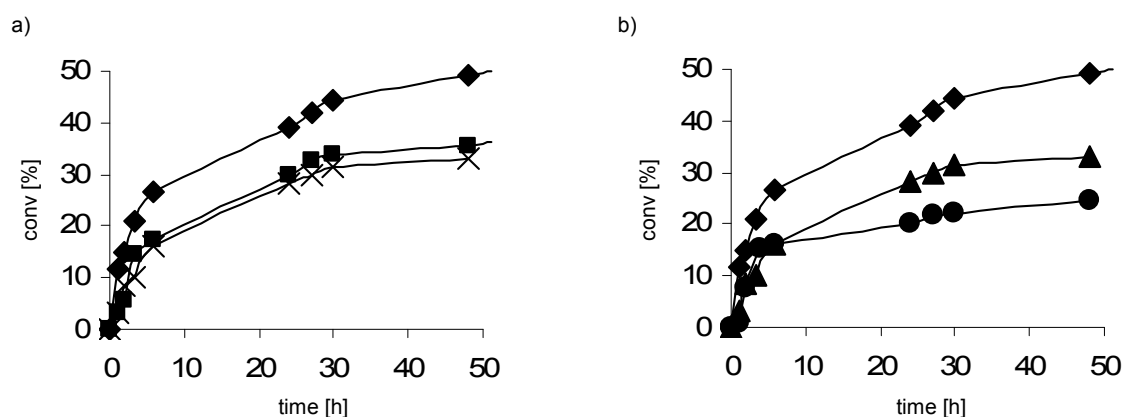


Figure 6. Effects of **1** (a) and HCN (b) on Novozym 435 ($2 \cdot 10^7$ U) in the kinetic resolution of (*R,S*)-**2** (0.1 M). Control reaction (♦), **1** 100 mM (■), **1** 300 mM (×), HCN 100 mM (▲), HCN 600 mM (●).

Both reactants partially inhibited the enzyme and in the reaction with HCN the effect was concentration dependent. Some reports indicate that HCN may inhibit Novozym 435 due to its acidity.²¹ we note, however, that HCN is a weak acid (pKa 9.37) that is unlikely to shift the pH beyond the enzyme's operational pH range (5 – 8). It is possible however that the inhibition occurred under a negative influence of polymerized HCN formed in contact with molecular sieves.²¹ Possibly, the inhibition of CALB might also be caused by HCN in combination with the reaction solvent. Adlercreutz group showed that HCN can have a strong influence on the activity of some enzymes, depending on hydrophobicity of the solvent (high log P). It was reported that solvents such as DIPE (log P 1.9) in the mixture with HCN may cause inhibition due to the limited HCN solubility in the reaction medium.²²

2.7. Asymmetric, one-pot chemo-enzymatic synthesis of (S)-mandelonitrile acetate with Novozym 435 and surfactants as the hydrocyanation and racemization catalyst

Since water is undesired in this reaction, the one-pot reactions were carried out with reactants dried over activated molecular sieves prior to the reaction. The highest conversion (4.7%) of (*R,S*)-**2** into (*S*)-**2** acetate was observed after 52 h with sorbitan sesquioleate, but with Triton X 100 or Tween 80 only 2.2% conversion was measured after the same reaction time (Figure 7). In the control reaction (no enzyme added) 2.5% (*R,S*)-**2** was unselectively acylated into the corresponding protected cyanohydrin. Hence, the contribution of CALB to the esterification is disputable.

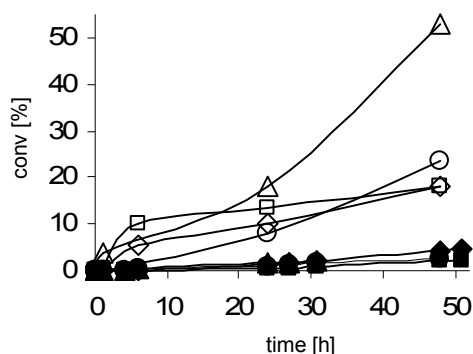


Figure 7. Asymmetric conversion of (*R,S*)-**2** (0.1 M) and isopropenyl acetate (0.3 M) into the corresponding (*S*)-**2** acetate with surfactants (20 mg) as the hydrocyanation and racemization catalyst and Novozym 435 ($2 \cdot 10^7$ U) as the acylation catalyst. **1** (open boxes), (*R,S*)-**2** (filled boxes) conversions: sorbitan sesquioleate (◇◆), Triton X 100 (□■), Tween 80 (△▲), test (○●).

Surprisingly, unlike in the separate reactions studied before, the one-pot reaction rates with surfactants and Novozym 435 were very slow. Additionally, the immobilize lifetime in the stirred reaction is limited to two days due to mechanical damage of the carrier. Traces of water released from the polymeric enzyme carrier can cause the reverse enzymatic reaction or hydrolysis of isopropenyl acetate that will result in formation of acetone and acetic acid. The latter is a recognized CALB inhibitor.²¹ Additionally, surfactants proved not to be good racemization catalysts. The concentration measurements of the particular **2** enantiomers revealed no progress of the racemization process (data not shown). It is possible that the surfactants activity loss was caused by the transesterification of isopropenyl acetate with the hydroxyl groups present in the surfactant molecules.

2.8. Asymmetric, one-pot chemo-enzymatic synthesis of (*S*)-mandelonitrile acetate with surfactants as the hydrocyanation catalysts. Reaction in the presence of tributylamine

As already mentioned in the above paragraphs, Novozym 435 pH optimum is in the range of pH 5 – 8, and acetic acid can readily bring the reaction medium beyond that region causing deactivation of the immobilize. To neutralize this effect, an organic base such as tributylamine was added into the one-pot reaction mixture (Figure 8).

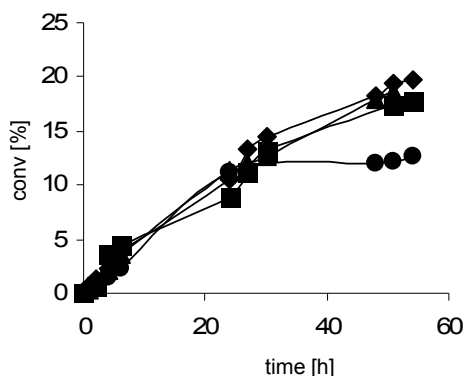


Figure 8. Asymmetric, one-pot conversion of **1** (0.1 M), HCN (0.6 M) and isopropenyl acetate (0.3 M) into the corresponding (*S*)-**2** acetate in the presence of Novozym 435 ($1 \cdot 10^7$ U), surfactants (20 mg) and tributylamine (5% v/v). Control reaction (no surfactant) (◆), sorbitan sesquioleate (■), Triton X 100 (▲), Tween 80 (●).

The addition of tributylamine indeed improved the asymmetric acylation of (*R,S*)-**2** and 18% of (*R,S*)-**2** (Tween 80, 12%) was converted into (*S*)-**2** acetate comparing to the previous max. 4.7% obtained in the reaction pictured in Figure 7. Surprisingly, the control reaction in which no hydrocyanation catalyst was present showed the same results. This indicated that the first hydrocyanation step can also be accelerated by tributylamine (Figure 9). Literature search revealed that some anion exchange columns containing tri-amine resins derivatives e.g. Amberlite® IRA-900 (a resin with tri-methylamine exchange sites) were demonstrated to be useful in hydrocyanation reactions.¹³

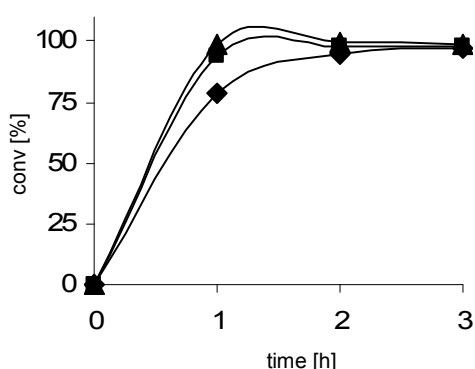


Figure 9. Hydrocyanation of **1** (0.1 M) with HCN (0.6 M) mediated by tributylamine. Tributylamine: 5% (◆), 10% (■), 20% (v/v) (▲).

1 was converted into the corresponding (*R,S*)-**2** in the presence of tributylamine, with a productivity of $5 \cdot 10^{-3} \text{ g g}^{-1} \text{ min}^{-1}$. That makes tributylamine the third fastest catalyst

(after Tween 80 and sorbitan sesquioleate, see Table 2). The amount of tributylamine (above 5% v/v) did not affect the hydrocyanation rate, indicating pH driven hydrocyanation. Further tests showed that unlike the surfactants, the catalytic properties of the base are not affected by isopropenyl acetate (data not shown). The following one-pot, two step synthesis experiments with 5%, 10% and 20% (v/v) tributylamine as hydrocyanation and racemization catalyst and Novozym 435 as asymmetric acylation catalyst (Figure 10) confirmed the hypothesis.

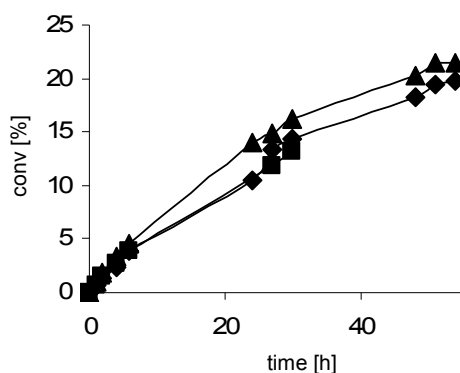


Figure 10. Asymmetric, two step, one-pot synthesis of (*S*)-**2** acetate from **1** (0.1 M), HCN (0.6 M) and isopropenyl acetate (0.3 M) and catalyzed by Novozym 435 ($1 \cdot 10^7$ U, not dried) and tributylamine. Tributylamine: 5% (◆), 10% (■), 20% (v/v) (▲).

In these experiments **1** was fully converted into the corresponding (*R,S*)-**2** in < 2h (data not shown). The acylation reaction progressed for 48 h and was terminated afterwards due to the mechanical damage of the immobilizate. 22% (*S*)-**2** was converted into the corresponding acetate. The chiral analyses showed a big disproportion between enantiomers of **2** indicating slow racemization (slower than the acylation reaction). The ee measurements in all cases showed > 98% (*S*)-**2** acetate. Experiments with double Novozym 435 load ($2 \cdot 10^7$ U) showed similar results.

2.9. Organic base and dried Novozym 435 catalyzed asymmetric, two step, one-pot synthesis of (*S*)-mandelonitrile acetate. Comparison of stirred and shaken reaction

As shown before, Novozym 435 carrier is crushed upon intensive mechanical stirring for a prolonged time. The crushed carrier can release water that can react with isopropenyl acetate to give acetic acid as one of the products. The latter in turn,

deactivates CALB. To prevent this occurrence, dried Novozym 435 was used in the asymmetric, two step, one-pot tests. Stirred and shaken reactions were compared (Figure 11a, b respectively).

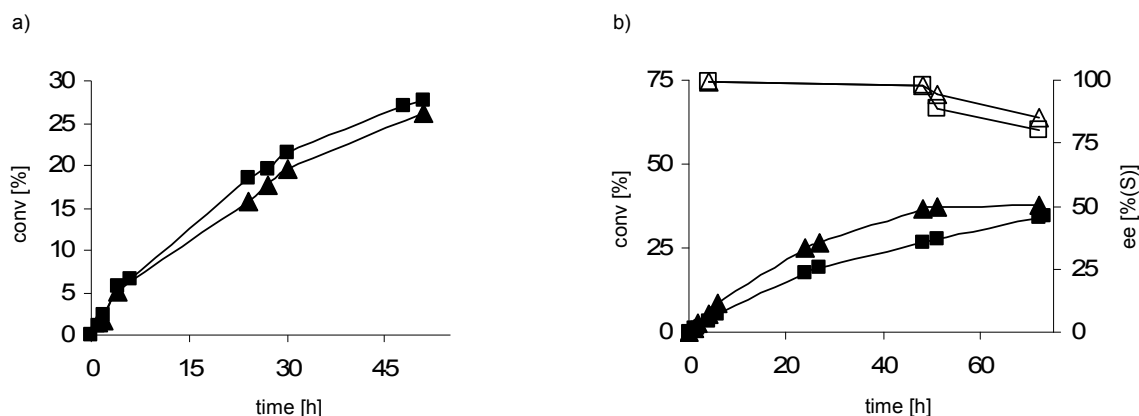


Figure 11. Asymmetric, two-step, one-pot tributylamine and Novozym 435 ($2 \cdot 10^7$ U, dried) catalyzed synthesis of (S)-2 acetate from **1** (0.1 M), HCN (0.6 M) and isopropenyl acetate (0.3 M). Conversions of (R,S)-2 (filled boxes) and (S)-2 acetate ee (open boxes) in; stirred reaction (a), orbital shaker (b) tributylamine; 10% (■), 20% (v/v) (▲).

The reactions with tributylamine (10 and 20% v/v) needed < 2 h to fully convert **1** into (R,S)-2. Shaking significantly improved the process. Unlike in the stirred reaction, no immobilizate mechanical damage was observed after two days of shaking. The measured acylation yield in the latter reaction system was higher (up to 39% after 51 h with 20% tributylamine). Unfortunately, the inefficient racemization system caused the accumulation of (R)-2 which when the concentration of the (S)-enantiomer decreased, became too competitive the enzymatic acylation reaction, causing the ee to decrease to < 79% after 74 h (Figure 11b).

3. Conclusions

Ionic and non-ionic surfactants showed a useful activity in the hydrocyanation of aldehydes and ketones, when applied in DIPE. Attempts to combine surfactant mediated reversible hydrocyanation with lipase catalyzed resolution of the cyanohydrin were not successful. Tributylamine likewise acted as hydrocyanation catalyst and could be combined with lipase catalyzed acylation of the cyanohydrin.

Unfortunately, the racemization of the non-reacting enantiomer was slow and dynamic kinetic resolution could not be accomplished.

4. Materials and methods

Chemicals

Semi-purified (S)-hydroxynitrile lyase from *Manihot esculenta* [E.C. 4.1.2.10] (protein content 88 mg mL⁻¹) was obtained from Julich Fine Chemicals (Jülich, Germany), Novozym 435 was from Novozymes (Bagsvæd, Denmark). Sodium dodecyl sulfate (SDS) was purchased from Merck, β -Octyl-glucoside (1-O-*n*-octyl- β -D-glucopyranoside), sorbitan sesquioleate, sorbitan monostearate, Triton X 100, molecular sieves 3Å (1.6 mm pellets) were from Sigma-Aldrich. PEG 8000, Tween 80, benzaldehyde (redistilled, +99%), phenylacetone, acetophenone, tributylamine (99%), isopropenyl acetate (99%), (*R,S*)-mandelonitrile (tech grade) were obtained from Acros. Sucrose monocaprate and sucrose monolaurate were from Dojindo, tetrabutyl ammonium chloride, DIPE, acrolein and veratrol (+99%) were from Fluka, cinnamaldehyde (+99%) was from Janssen Chimica. All the surfactants were used as provided by suppliers. HCN was prepared as previously described.¹¹

Instrumentation

Conversions of the reactants were monitored by straight phase HPLC analysis, using a Waters 625 LC pump, a 0.46 $\times$ 250 mm CHIRALCEL[®] OB-H column and a Waters 486 UV detector at 215 nm; eluant hexane-isopropyl alcohol (90:10, v/v) at 0.5 mL min⁻¹.

Molecular sieves activation

A portion of molecular sieves were placed in an oven and dried at 300 °C for 12 h (with 5 °C min⁻¹ heating and cooling rate). The activated drying agent was stored in a desiccator under vacuum.

SDS catalyzed hydrocyanation in buffered media (0 – 2% buffer)

SDS (5 mg) was weighed into a 2 mL PTFE septum glass vial and DIPE (kept on 20 mM citrate buffer pH 5.5), 20 mM citrate, buffer pH 5.5 and 0.1 mL (0.10 mg (7.8 U)) *M. esculenta* (S)-hydroxynitrile lyase CLEA suspension were added. HCN (70 mM) and benzaldehyde (10 mM) were added to start the reactions. The final reaction volume was 1 mL. The reactions were magnetically stirred at 25 °C. Samples were withdrawn to measure the reaction progress.

Surfactant productivity tests

The productivity [$\text{g g}^{-1} \text{ min}^{-1}$] is defined as the amount of product in mg that is produced by 1 mg of catalyst in 1 min.

All surfactants but sorbitan sesquioleate (10 mg), Tween 80 (20 mg) were weighed in 50 mg portions in 2 mL PTFE septum glass vials. DIPE, HCN (70 mM) were added. Benzaldehyde (10 mM) was added to start the reaction. The final reaction volume was 1 mL. All the reactants and the medium were dried with molecular sieves. The reactions were magnetically stirred at 25 °C. Samples were withdrawn to measure the reaction progress.

Hydrocyanation of aldehydes and ketones

Sorbitan sesquioleate and Tween 80 were weighed in 50 mg portions in 2 mL PTFE septum glass vials (acrolein hydrocyanation, 1 mg). DIPE and HCN (60 mM) were added. Benzaldehyde, acrolein, phenyl acetone, acetophenone and cinnamic aldehyde (10 mM) were added to start the reaction. The final reaction volume was 1 mL. All the reactants and the medium were dried with molecular sieves. The reactions were magnetically stirred at 25 °C. Samples were withdrawn to measure the reaction progress.

Inhibition of surfactant catalyzed hydrocyanation by isopropenyl acetate

20 mg Portions of sorbitan sesquioleate, Triton X 100 and Tween 80 were weighed into a 2 mL PTFE septum glass vials. DIPE, isopropenyl acetate (0.3 M), HCN (0.6 M) and veratrol (10 mM) were added. Finally, benzaldehyde (0.1 M) was added to start the test. The final reaction volume was 1 mL and it was magnetically stirred at 25 °C. All reaction ingredients and the medium were dried with molecular sieves beforehand. Samples were withdrawn after 1 h to measure the reaction progress.

Effect of the isopropenyl acetate concentration on Triton X 100 catalyzed hydrocyanation

20 mg Triton X 100 was weighed into 2 mL PTFE septum glass vials. DIPE, isopropenyl acetate (0.3 and 0.6 M), HCN (0.6 M) and veratrol (10 mM) were added. Finally, benzaldehyde (0.1 M) was added to start the test. The final reaction volume was 1 mL and it was magnetically stirred at 25 °C. All reaction ingredients and the medium were dried with molecular sieves. Samples were withdrawn to measure the reaction progress.

Effects of the benzaldehyde and HCN concentration on Novozym 435 catalyzed kinetic resolution of (*R,S*)-mandelonitrile

100 mg ($2 \cdot 10^7$ U) Novozym 435 was weighed into a 2 mL PTFE septum glass vial. DIPE, HCN (0.1 and 0.6 M), benzaldehyde (0.1 and 0.3 M), veratrol (10 mM) and isopropenyl acetate (0.3 M) were added. Finally, (*R,S*)-mandelonitrile (0.1 M) was added to start the reaction. The final reaction volume was 1 mL and it was magnetically stirred at 25 °C. All reaction ingredients and the medium were dried with molecular sieves. Samples were withdrawn to measure the reaction progress and enantiopurities.

Asymmetric, two step, one-pot synthesis of (*S*)-mandelonitrile acetate with surfactants and Novozym 435

100 mg ($2 \cdot 10^7$ U) Novozym 435 (dried for 0.5 h over P_2O_5 under vacuum) was weighed into a 2 mL PTFE septum glass vial. DIPE, 20 mg surfactant, isopropenyl acetate (0.3 M) and veratrol (10 mM) were added. Finally, (*R,S*)-mandelonitrile (0.1 M) was added to start the test. The final reaction volume was 1 mL and was magnetically stirred at 25 °C. All reaction ingredients and the medium were dried with molecular sieves beforehand. Samples were withdrawn to measure the reaction progress and enantiopurities.

One-pot chemo-enzymatic synthesis of (*S*)-mandelonitrile acetate with surfactants as the hydrocyanation catalysts. Reaction in the presence of tributylamine

50 mg ($1 \cdot 10^7$ U) Novozym 435 was weighed into a 2 mL PTFE septum glass vial. DIPE, 20 mg surfactant, isopropenyl acetate (0.3 M), tributylamine (5% v/v), HCN (0.6 M) and veratrol (10 mM) were added. Finally, benzaldehyde (0.1 M) was added to start the test. The final reaction volume was 1 mL and it was magnetically stirred at

25 °C. All reaction ingredients and the medium were dried with molecular sieves. Samples were withdrawn to measure the reaction progress and enantiopurities.

Benzaldehyde hydrocyanation with tributylamine

A 2 mL PTFE septum glass vial was charged with DIPE, HCN (0.6 M), veratrol (10 mM) and different volumes of tributylamine (5, 10 and 20% v/v). Finally, benzaldehyde (0.1 M) was added to start the test. The final reaction volume was 1 mL and it was magnetically stirred at 25 °C. All reaction ingredients and the medium were dried with molecular sieves. Samples were withdrawn to measure the reaction progress.

DKR of (*R,S*)-mandelonitrile and tributylamine with Novozym 435 as the racemization catalyst

50 mg ($1 \cdot 10^7$ U, not dried) Novozym 435 was weighed into a 2 mL PTFE septum glass vial. DIPE, different volumes of tributylamine (5, 10 and 20% v/v), isopropenyl acetate (0.3 M), HCN (0.6 M) and veratrol (10 mM) were added. Finally, benzaldehyde (0.1 M) was added to start the test. The final reaction volume was 1 mL and it was magnetically stirred at 25 °C. All reaction ingredients and the medium were dried with molecular sieves beforehand. Samples were withdrawn to measure the reaction progress and enantiopurities.

One-pot chemo-enzymatic synthesis of (*S*)-mandelonitrile acetate with tributylamine as the hydrocyanation catalysts. Comparison of stirred and shaken reaction

100 mg ($2 \cdot 10^7$ U) Novozym 435 was weighed into a 2 mL PTFE septum glass vial. DIPE, tributylamine (5, 10 and 20% v/v), HCN (0.6 M), isopropenyl acetate (0.3 M) and veratrol (10 mM) were added. Finally, benzaldehyde (0.1 M) was added to start the test. The final reaction volume was 1 mL and it was magnetically stirred or shaken at 25 °C. All reaction ingredients and the medium were dried with molecular sieves. Samples were withdrawn to measure the reaction progress and enantiopurities.

5. Reference list

1. Hawkins, J., Chadwick, P., Messenger, E. T., and Lykke, M. Stabilized Enzyme Dispersion. EP19890306974 [EPO351162]. 1989.
2. Sheldon, R. A.; van Rantwijk, F. *Aust. J. Chem.* **2004**, *57*, 281-289.
3. Texter, J. In *Encyclopedia of Chemical Physics and Physical Chemistry*; Moor, J. H.; Spenser, N. D. Eds. Micelles. IoP: 2001; pp. 2285-2316.
4. Eicke, H. F.; Christen, H. *J. Colloid Interface Sci.* **1974**, *46*, 417-436.
5. Rispens, T.; Engberts, J. B. F. N. *J. Org. Chem.* **2002**, *67*, 7369-7377.
6. van den Broeke, L. J. P.; de Bruijn, V. G.; Heijnen, J. H. M.; Keurentjes, J. T. F. *Ind. Eng. Chem. Res.* **2001**, *40*, 5240-5245.
7. Zhil'tsova, E. P.; Kudryavtseva, L. A.; Mikhailov, A. S.; Semenov, V. E.; Reznik, V. S.; Konovalov, A. I. *Russ. J. Gen. Chem.* **2008**, *78*, 50-56.
8. Thoman, C. J.; Habeeb, T. D.; Huhn, M.; Korpusik, M.; Slis, D. F. *J. Org. Chem.* **1989**, *54*, 4476-4478.
9. Volkov, A. G. *Interfacial Catalysis*; CRC Press: 2002.
10. Jackson, W. R.; Jayatilake, G. S.; Matthews, B. R.; Wilshire, C. *Aust. J. Chem.* **1988**, *41*, 203-213.
11. Chmura, A.; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1655-1661.
12. Veum, L.; Hanefeld, U. *Synlett* **2005**, 2382-2384.
13. Inagaki, M.; Hiratake, J.; Nishioka, T.; Oda, J. *J. Am. Chem. Soc.* **1991**, *113*, 9360-9361.
14. Zhang, T.; Yang, L.; Zhu, Z.; Wu, J. *J. Mol. Catal. B: Enzym.* **2002**, *18*, 315-323.
15. North, M. *Tetrahedron: Asymmetry* **2003**, *14*, 147-176.
16. Veum, L.; Hanefeld, U. *Tetrahedron: Asymmetry* **2004**, *15*, 3707-3709.
17. Hanefeld, U.: *Org. Biomol. Chem.* **2003**, *1*, 2405-2415.
18. Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis. Regio- and Stereoselective Biotransformations*; Wiley-VCh: 1999.
19. Heinsman, N. W. J. T.; Schroen, C. G. P. H.; Padt van der, A.; Franssen, M. C. R.; Boom, R. M.; Riet van't, K. *Tetrahedron: Asymmetry* **2003**, *14*, 2699-2704.
20. Lau, R. M.; Sorgedrager, M.; Carrea, G.; van Rantwijk, F.; Secundo, F.; Sheldon, R. A. *Green Chem.* **2004**, *6*, 483-487.

21. Veum, L.; Kanerva, L. T.; Halling, P. J.; Maschmeyer, T.; Hanefeld, U. *Adv. Synth. Catal.* **2005**, *347*, 1015-1021.
22. Costes, D.; Wehtje, E.; Adlercreutz, P. *Enzyme Microb. Technol.* **1999**, *25*, 384-391.

Summary

Today, enzymes are widely used industrial catalysts. Driven by the trend to shorter and technological simpler procedures, there is an increasing interest in one-pot, multi-enzyme reactions. Designing these is challenging, in particular with free enzymes, because the reaction conditions (such as pH, tolerance of organic solvents etc.) of the enzymes may be incompatible.

Following the recent trend in the synthesis of α -hydroxy acids to apply asymmetric enzymatic transformations, the main scope of the Thesis is to develop a one-pot, multi-enzymatic pathway to synthesize enantiomerically pure α -hydroxy acids using enzymes from two different classes, an oxynitrilase (a lyase) and a nitrilase (a hydrolase). The course of the investigations fortuitously led us to new subjects, such as surfactant catalyzed hydrocyanation and the discovery of a new nitrilase.

Chapter 1 provides a general introduction to enantiomerically pure α -hydroxy acids and to the methods used for their synthesis. It overviews the application areas of these compounds and describes in detail some of the commercial production processes for pharmaceuticals based on enantiomerically pure α -hydroxy acids. In the following part of this Chapter, a more detailed explanation is given about how scientists copy Nature to synthesize α -hydroxy acids. Finally, an introduction to nitrilases is given in historical and application context.

Chapter 2 focuses on the hydrocyanation reactions of various aldehydes and ketones in microaqueous medium, catalysed by a cross-linked enzyme aggregate (CLEA) of the oxynitrilase from *Manihot esculenta*. The immobilizate proved to be extremely stable under the reaction conditions, resisting high reactant concentrations and very high di-isopropylether contents. The enzyme remained active even in pure organic solvent in the absence of a discrete buffer phase.

In most cases, using our microaqueous bi-phasic approach, we obtained products with higher enantiopurities than those reported in literature. We also found that enzymatic cycling of reactant and product causes a decay of the product ee upon the approach of reaction equilibrium.

Chapter 3 is concerned with the main goal of this thesis: We successfully prepared a co-immobilizate of *Manihot esculenta* oxynitrilase and *Pseudomonas fluorescens* nitrilase in the form of a combi-CLEA. Under the cross-linking conditions applied the activity recovery was high for both enzymes. The immobilizate also showed excellent recyclability properties and high operational stability with the substrate concentrations (0.25 M). Benzaldehyde was converted into nearly enantiomerically pure (S)-mandelic acid, demonstrating that the bienzymatic methodology is basically sound and that racemisation of the intermediate nitrile can be avoided. We also showed that the combi-CLEA gave better results in terms of enantiopurity than in reactions with two separate CLEAs. The process however, is disturbed by the unexpected formation of large amounts of (S)-mandelic amide.

In **Chapter 4** we introduce the concept of a triple-CLEA as a counter-measure to the problem of amide formation as a byproduct from the double activity of EBC191 *Pseudomonas fluorescens* nitrilase in the hydrolysis of (S)-mandelonitrile. The screening for suitable amide hydrolyzing enzymes revealed MP50 amidase to be most suitable from all the enzymes tested. The three enzymes were successfully co-immobilized in the form of a triple-CLEA. A method to assay the activities of the different enzymes in the three-enzyme immobilizate was developed.

The rationally designed triple-CLEA was successfully used in the synthesis of (S)-mandelic acid. In the reaction, the co-immobilized MP50 amidase simultaneously hydrolysed the formed (S)-mandelamide into the corresponding hydroxy acid. We found that more activity can be recovered in the triple-CLEA by aggregating the enzymes one by one, starting with the most sensitive ones. Presumably, a coating effect is observed here. The storage stability of triple-CLEA in the reaction (citrate) buffer was not satisfactory but was hugely improved by storage in sat. $(\text{NH}_4)_2\text{SO}_4$ at pH 8.7.

Chapter 5 describes a competitor to the combi-CLEA approach, a whole-cell biocatalyst based on an *E. coli* that simultaneously expresses the oxynitrilase as well as the nitrilase. In synthetic tests the resulting double clone demonstrated that it is an efficient catalyst for synthesizing (S)-mandelic acid. We also demonstrated that the double clone is more efficient in aqueous buffer than in an aqueous-organic biphasic system. Unfortunately, when applying KCN as the cyanide donor, pH control is

rapidly lost and the resulting pH shift towards alkaline defeats the whole purpose of the bi-enzymatic procedure. The biphasic system, in contrast, although slower, showed to be more suitable in reactions with high concentration of reactants. We also found that the acid/ amide ratio is strongly dependent on the reactant concentration and pH.

In **Chapter 6** a detailed investigation into the amide formation in the presence of *Pseudomonas fluorescens* nitrilase is described. We have shown that the latter enzyme converts nitriles into the carboxylic acid as well as the amide and that the relative formation of acid and amide is subject to the pH and the temperature. We have found that electron-withdrawing substituents at the α -position favor amide formation. We have also shown, for the first time, that the absolute configuration at the α -position in the reactant exerts a dramatic influence on the extent of amide formation. Based on the experimental data, we have proposed the possible mechanism that can steer the nitrile to be transformed into either the corresponding acid or amide.

Chapter 7 entirely focuses on the characterisation of *Halomonas nitrilicus* nitrilase. We show that the latter organism, employed as a whole-cell biocatalyst, hydrolyses nitriles via the nitrilase pathway. The corresponding carboxylic acid is nearly exclusively produced, with negligible amide formation. We also show that the nitrilase has a quite relaxed substrate specificity, with a preference for phenylacetone nitrile derivatives. Its potential was demonstrated in a chemoenzymatic dynamic kinetic resolution of mandelonitrile, which afforded (*R*)-mandelic acid with 95% enantiomeric purity. A fed-batch hydrolysis of phenylacetone nitrile resulted in 0.84 M phenylacetic acid.

Attempts to extract *Halomonas nitrilicus* nitrilase from the cells proved to be far from trivial and is further complicated by its sensitivity for oxygen. From our, somewhat tentative, experiments it would seem that osmotic shock could be developed into an efficient procedure.

Typically, in enzymatic reactions, surfactants are used to disperse enzymes in the reaction phase.

In **Chapter 8** we report that ionic and non-ionic surfactants show a useful activity in the hydrocyanation of aldehydes and ketones, when applied in di-isopropylether.

Based on this discovery, we also designed a chemo-enzymatic combining surfactant mediated reversible hydrocyanation with lipase catalyzed resolution of the cyanohydrin to produce enantio-pure protected cyanohydrin. The tests were not successful, however. Tributylamine likewise acted as hydrocyanation catalyst and could be combined with lipase catalyzed acylation of the cyanohydrin. Unfortunately, the racemization of the non-reacting enantiomer was slow and dynamic kinetic resolution could not be accomplished.

Samenvatting

Vandaag de dag worden enzymen veelvuldig toegepast als industriële katalysatoren. Gedreven door de trend om kortere en technologisch eenvoudigere reactieprocedures te ontwikkelen is er een toenemende interesse in éénpots multienzymatische reacties ontstaan. Het ontwerpen van dit soort reacties is een uitdaging. In het bijzonder in het geval van vrije enzymen, omdat de reactiecondities (zoals pH, tolerantie voor organische oplosmiddelen, enz.) van de diverse enzymen onverenigbaar kunnen zijn.

Vanwege de recente trend om voor de synthese van α -hydroxycarbonsuren asymmetrische enzymatische omzettingen te gebruiken is het hoofdonderwerp van dit proefschrift het ontwikkelen van een éénpots multienzymatische reactie om enantiozuivere α -hydroxycarbonsuren te synthetiseren. Hiervoor wordt gebruik gemaakt van enzymen uit twee verschillende klassen: een oxynitrilase (een lyase) en een nitrilase (een hydrolase). Gedurende het onderzoek hebben we ons ook georiënteerd op nieuwe onderwerpen zoals hydrocyanering gekatalyseerd door oppervlakteactieve stoffen en de ontdekking van een nieuw nitrilase.

Hoofdstuk 1 begint met een algemene introductie over enantiozuivere α -hydroxycarbonsuren en over de methoden die gebruikt worden voor de synthese van deze stoffen. Het hoofdstuk geeft een overzicht van de toepassingsgebieden van deze verbindingen en beschrijft in detail enkele commerciële productieprocessen voor medicijnen die gebaseerd zijn op enantiozuivere α -hydroxycarbonsuren. In het tweede gedeelte van dit hoofdstuk wordt een meer gedetailleerde uitleg gegeven over hoe wetenschappers trachten om enantiozuivere α -hydroxycarbonsuren te synthetiseren door natuurlijke processen te kopiëren. Ten slotte wordt een introductie gegeven over nitrilases in een historische en toepassingsgerichte context.

Hoofdstuk 2 richt zich op hydrocyaneringsreacties van verschillende aldehydes en ketonen gekatalyseerd door het oxynitrilase uit *Manihot esculenta* in de vorm van een gecrosslinked enzymaggregaat (CLEA) in organisch oplosmiddel met een minimale (< 1%) bufferfase. Het geïmmobiliseerde enzym bleek zeer stabiel te zijn onder reactiecondities met hoge substraatconcentraties en zeer grote hoeveelheden

diisopropylether. Het enzym behield zelfs zijn activiteit in puur organisch oplosmiddel bij het ontbreken van een discrete bufferfase.

In de meeste gevallen werden, door gebruik te maken van onze minimale bufferfase, producten verkregen met hogere enantiozuiverheden dan eerder beschreven in de literatuur. We ontdekten ook dat een enzymatisch gekatalyseerd evenwicht tussen substraat en product bij het benaderen van het reactie-evenwicht een verslechtering van de ee van het product veroorzaakte.

Hoofdstuk 3 behandelt het hoofddoel van dit proefschrift. We hebben met succes een coïmmobilisaat van *Manihot esculenta* oxynitrilase en *Pseudomonas fluorescens* nitrilase bereid in de vorm van een combi-CLEA. De activiteitsherwinning onder de toegepaste crosslinkingcondities was hoog voor beide enzymen. Het immobilisaat bleek ook uitstekende eigenschappen voor hergebruik te hebben, evenals hoge operationele stabiliteit bij de gebruikte substraatconcentraties (0.25 M).

Benzaldehyde werd omgezet in praktisch enantiozuiver (S)-amandelzuur, wat aantoont dat de bienzymatische methodologie in essentie een succes is en dat racemisatie van het nitril tussenproduct kan worden voorkomen. We hebben ook aangetoond dat gebruik van de combi-CLEA betere resultaten gaf met betrekking tot de enantiozuiverheid dan het gebruik van twee aparte CLEAs. Een nadeel van dit proces is echter de onverwachte vorming van grote hoeveelheden (S)-mandelamide.

In **Hoofdstuk 4** introduceren we het concept van de triple-CLEA als een maatregel om het probleem van de amidevorming als bijproduct van de dubbele activiteit van EBC191 *Pseudomonas fluorescens* nitrilase in de hydrolyse van (S)-mandelonitril op te lossen. Uit een screening voor geschikte amidehydrolyserende enzymen bleek dat MP50 amidase het meest geschikte enzym was. De drie enzymen werden met succes gecoïmmobiliseerd in de vorm van een triple-CLEA. Ook werd een methode ontwikkeld om de activiteiten van de verschillende enzymen in dit immobilisaat met drie enzymen te bepalen.

De rationeel ontwikkelde triple-CLEA werd vervolgens met succes toegepast in de synthese van (S)-amandelzuur. In de reactie werd het gevormde (S)-mandelamide nu tegelijkertijd omgezet in het overeenkomstige carbonzuur door het gecoïmmobiliseerde MP50 amidase. We ontdekten dat meer enzymactiviteit kon worden herwonnen in de triple-CLEA door de enzymen één voor één te aggregeren,

beginnende met de meest kwetsbare enzymen. Vermoedelijk observeren we hier een coatingeffect. De opslagstabiliteit van de triple-CLEA in de reactiebuffer (citraat) was niet bevredigend maar verbeterde substantieel bij opslag in verzadigd ammoniumsulfaat van pH 8.7.

Hoofdstuk 5 beschrijft een concurrent van de combi-CLEA aanpak: een hele cel biokatalysator gebaseerd op een *E. coli* die tegelijkertijd een oxynitrilase en een nitrilase tot expressie brengt. In synthetische tests werd aangetoond dat de dubbele kloon een efficiënte katalysator is voor de synthese van (S)-amandelzuur. Ook toonden we aan dat de dubbele kloon efficiënter is in een waterige buffer dan in een tweefasensysteem met water en een organisch oplosmiddel. Helaas wordt de controle van de pH snel verloren als KCN als cyanide donor wordt gebruikt en wordt het hele doel van het gebruik van een bienzymatische procedure tenietgedaan door de resulterende basische pH verschuiving. In tegenstelling tot het éénfasensysteem was het tragere tweefasensysteem beter geschikt voor gebruik bij hoge substraatconcentraties. Ook werd ontdekt dat de zuur/amide verhouding sterk afhankelijk is van de concentratie van de substraten en de pH.

In **Hoofdstuk 6** wordt een gedetailleerd onderzoek naar de amidevorming in de aanwezigheid van *Pseudomonas fluorescens* nitrilase beschreven. We hebben aangetoond in dit hoofdstuk dat het enzym nitrillen in zowel carbonzuren als amides omzet en dat de relatieve hoeveelheid zuur en amide dat gevormd wordt afhankelijk is van de pH en de temperatuur. We hebben gevonden dat elektron zuigende substituenten op de α -positie gunstig zijn voor de vorming van het amide. We hebben ook voor de eerste keer aangetoond dat de absolute configuratie op de α -positie van het substraat een enorme invloed uitoefent op de hoeveelheid amide dat gevormd wordt. Gebaseerd op de experimentele data hebben we een mogelijk mechanisme voorgesteld dat verklaart waarom uit het nitril zowel het overeenkomstige amide als het zuur gevormd kan worden.

Hoofdstuk 7 concentreert zich volledig op de karakterisatie van het nitrilase uit *Halomonas nitrilicus*. We tonen aan dat nitrillen via de nitrilase gekatalyseerd reactie worden omgezet wanneer dit organisme wordt toegepast als hele cel biokatalysator. Bijna uitsluitend het overeenkomstige carbonzuur wordt geproduceerd met een verwaarloosbare hoeveelheid amide. We tonen ook aan dat het nitrilase een vrij

brede substraatspecificiteit heeft met een voorkeur voor fenylacetonitrilachtige verbindingen. Het potentieel van dit enzym werd aangetoond door 95 % enantiozuiver (*R*)-amandelzuur te produceren in een chemisch-enzymatische dynamisch kinetische resolutie van mandelonitril. Een fed-batch hydrolyse van fenylacetonitril resulteerde in 0.84 M fenylazijnzuur.

Bij pogingen om het *Halomonas nitrilicus* nitrilase te isoleren uit de hele cellen bleek dat dit verre van triviaal was. Dit proces werd verder gehinderd door de gevoeligheid van het enzym voor zuurstof. Uit onze voorlopige experimenten zou geconcludeerd kunnen worden dat het gebruik van de osmotische schok techniek verder ontwikkeld zou kunnen worden voor een efficiënte procedure.

Over het algemeen worden oppervlakteactieve stoffen gebruikt om enzymen te disperseren in de reactiefase.

In **Hoofdstuk 8** tonen we aan dat ionische en niet-ionische oppervlakteactieve stoffen, wanneer deze toegepast worden in diisopropylether, een bruikbare activiteit vertonen in de hydrocyanering van aldehydes en ketonen. Gebaseerd op deze ontdekking hebben we in dit hoofdstuk ook getracht om een chemoenzymatische resolutie te ontwerpen voor de productie van enantiozuivere beschermde cyaanhydrinen. Deze chemoenzymatische resolutie combineert omkeerbare hydrocyanering, veroorzaakt door de oppervlakteactieve stof, met een lipase gekatalyseerde resolutie van het cyanohydrin. De experimenten waren echter niet succesvol. Tributylamine kon op de zelfde manier als hydrocyaneringskatalysator worden gebruikt in de gecombineerde reactie met lipase gekatalyseerde acylering van het cyanohydrin. Helaas was de racemisatie van het niet reagerende enantiomeer langzaam en kon een dynamisch kinetische resolutie niet tot stand worden gebracht.

Acknowledgements

I would like to use the last pages of this book to personally thank all the people who have directly or indirectly contributed to my thesis.

First of all I would like to thank my promoter, Roger Sheldon. Roger, you gave me the chance to do my PhD research in your group and I am very grateful to you for the freedom you gave me with my research topic.

Fred, I would like to thank you for your patience in waiting for this thesis to be finalized. I am very glad you were willing to guide me throughout the PhD project. I greatly admire your knowledge and expertise. I think there is no chemical problem that is beyond your capability.

Mieke, thank you for your support with all of the administrative tasks, as well as countless of other matters. You are the oil that makes the group run smoothly.

I greatly acknowledge my project partners from the Institut für Mikrobiologie, Universität Stuttgart, with whom I had the pleasure to work on this PhD project.

Special thanks go to Professor Andreas Stolz for the opportunity to work in his laboratory.

Sven, Jan, Olga, Steffi, and Sibylle, thank you for your hospitality and for being such great colleagues. Sven and Jan, thank you for giving me a hand in the lab, for being excellent work mates, and for being good company while exploring the variety of lunch places around the university. Steffi, Olga, and Sibylle, thanks for all the discussions we had and for the nice daily treat. Coffee and spicy chocolate really is a superb combination! Steffi, “Sweeney Todd” did not become my favourite movie but I will surely not forget it.

I would like to thank Dimitry Sorokin for giving me the opportunity to work with you. Thanks to you my research was more versatile and thus even more interesting. Our collaboration resulted in enough material for a chapter and an interesting publication.

Linda, thank you for your help with growing some of the bacteria described in this thesis. Your untiring and cheerful personality, as well as your infectious enthusiasm, is a gift.

Luuk, you were the one who introduced me practically to my project. Thanks for that! I will remember you as “*The HCN Master*”. I always liked your friendly attitude, sense of humour, and patience. I am also grateful to you for the corrections and suggestions you made in some of the chapters in this thesis. Of course, I should not forget to thank you, as well as Van Vliet for vaporising the BBQ gear and all the other flammable materials after one of the memorable parties at the Delftse Hout. This way we did not have much to carry back home.

I would like to thank the following students who chose to work with me on some fragments of my PhD project: Adem Celik, Floris van der Zwan, and Jean-Luc Mession. Working with you guys was a pleasure and I hope you felt the same! If you read this thesis, you will surely find your contribution there!

Although it is difficult to manage in one paragraph, I would like to take the opportunity to thank all my BOC and other TU Delft colleagues, as well as those who came to work in BOC for shorter or longer periods. César (for laying the foundations of my research project), Seda (for all the fun in the lab and “the China experience”), Inga (for the initiated known as “*Inga-Pinga*”, for all the chats about life), Hilda, Anne, Michiel, Rob, Aida, Menno, Bruno (for introducing me to the world of nitrilases and for the opportunity to share some of the research topics), Aleksandra (dzięki za miłe sąsiedztwo w labie i zawsze ciekawe pogadanki w czasie czasami niekończących się dni), Pedro, Małgorzata, Isabel C., Daniela, Hans Peter (“*HP*”), Maria, Daniel (“*Dani*”), Marco N. & Marco C., David, Franja, Joelle, Martina, David K., Christophe (for the times we enjoyed Greek food together while chatting about nearly everything), Atsushi, Tsune, Antonio, Antonia, Paco, Arek, Silvia, Ksenia, Matthieu, Fabien, Zbigniew (dzięki za pomoc!), Jarle, Monica, Ton, Dirk, Chrétien, Selvedin, Lars, Jeroen, Daniel, Luigi, Tim, Marco, Remco, Leen M., Ulf H., Frank H., Isabel A., Kristina (for your assistance with the NMR measurements), Lars (for the nice chats and for keeping our HPLCs alive, without which I am sure I would still be working on my project until today) and others that I might forget to mention here.

Special thanks go to my office mates and my paranympths John and Sander. Guys, I could easily fill a couple of pages reminiscing all the things we did together while sharing the office and the lab, visiting all the conferences and meetings, and of course during numerous events outside the lab.

John, for me you are a good colleague and friend. You are an example of a real scientific passionate and you did not keep this passion only for yourself. I am grateful to you for your real interest in my project as well as for your help with organic syntheses and for all the discussions and advice. I have always thought you could be a good university teacher.

Sander, you are the last one to be mentioned in this part of the acknowledgements for a good reason. We managed to become friends. Thanks for that! It was great fun to go with you to places like the USA (I hope lobster is back on your menu...), China (an unforgettable adventure holiday!) and many others. Additionally, we could share some elements and therefore some problems of our PhD projects, which eventually led to a paper we published together. I am happy for you and your new relationship! All the best!

Dr. Peter Lankhorst. Because of the internship in your lab at DSM, as well as the experience and the way of working you thought me, I decided to go for a PhD. Thank you for that!

My friends in Leiden: Thierry, Merce and Fernando. Thierry, you are a good friend. I loved our any-time BBQ sessions in the garden and I am still astonished by your cooking talent! I hope you have a good time there in Heidelberg. Good luck with your plans! Merce, good luck with your plans too! Certainly, Barcelona is one of the places one does not want to leave for a longer period! Fernando, you earned your trip around the world and I hope it goes well. I cannot wait for you to tell us your tale when we meet again!

Finally, I would like to express my gratitude to my parents, mama and tata. Dziękuję Wam za wszystko co mi daliście i za to czego zdołali nauczyć. Bez waszego rodzicielskiego wsparcia nie byłbym dzisiaj kim jestem.

And last but not least to my beloved wife Sylwia. Dziękuję Ci za twoją wyrozumiałość, pomoc i wszystkie chwile, które razem przeżyliśmy. Kocham Cię.

List of publications

1. **Chmura, A.**; van der Kraan, G. M.; Kielar, F.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 1655-1661.
2. **Chmura, A.**; Shapovalova, A. A.; van Pelt, S.; van Rantwijk, F.; Tourova, T. P.; Muyzer, G.; Sorokin, D. Y. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 371-378.
3. Fernandes, B. C. M.; Mateo, C.; Kiziak, C.; **Chmura, A.**; Wacker, J.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Adv. Synth. Catal.* **2006**, *348*, 2597-2603.
4. Mateo, C.; **Chmura, A.**; Rustler, S.; van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *Tetrahedron: Asymmetry* **2006**, *17*, 320-323.
5. Rustler, S.; Muller, A.; Windeisen, V.; **Chmura, A.**; Fernandes, B. C. M.; Kiziak, C.; Stolz, A. *Enzyme Microb. Technol.* **2007**, *40*, 598-606.
6. Rustler, S.; **Chmura, A.**; Sheldon, R. A.; Stolz, A. *Stud. Mycol.* **2008**, *61*, 165-174.
7. Sosedov, O.; Matzer, K.; Burger, S.; Kiziak, C.; Baum, S.; Altenbuchner, J.; **Chmura, A.**; van Rantwijk, F.; Stolz, A. *Adv. Synth. Catal.* **2009**, *351*, 1531-1538.
8. van Rantwijk, F.; Mateo, C.; **Chmura, A.**; Fernandes, B.; Sheldon, R. A. In *Modern Biocatalysis: Stereoselective and Environmentally Friendly Reactions*; W.-D. Fessner; T. Anthonsen Eds. Nitrilases in the Enantioselective Synthesis of α -Hydroxycarboxylic Acids. Wiley-VCH: 2009; pp. 261-272.
9. **Chmura, A.**; et al., The combi-CLEA approach: cascade synthesis of enantiomerically pure (S)-mandelic acid – **in preparation**.

Oral presentations

1. COST D25 meeting on **Nitrile- and Amide-hydrolyzing enzymes as Tools in Organic Chemistry**, Delft, The Netherlands, July 2005.
2. Conference on **Multistep Enzyme Catalyzed Processes 2006**, Graz, Austria, April 2006.
3. NWO conference on **Synthetic Chemistry, Structural Synthesis and Bio-molecular Chemistry**, Lunteren, The Netherlands, October 2006.
4. Minisymposium on **Biocatalysis**, Hamburg, Germany, June 2007.
5. Minisymposium on biocatalysis at **Novozymes**, Copenhagen, Denmark, June 2007.
6. **International Symposium on Advanced Biological Engineering and Science**, Tsinghua University, Beijing, China, May 2008.

Curriculum vitae

Andrzej Chmura was born on the 3rd of May 1977 in Łańcut, Poland. Between 1997 and 2002 he studied Chemical Technology at the Politechnika Wrocławska in Wrocław, Poland. He graduated in 2003 with a specialisation in Fuels and Energy. Earlier, in 2002, he started a one-year graduation engineering program at the Hogeschool Zeeland, Vlissingen, The Netherlands. He spent the last six months of this graduation program at DSM Gist in Delft, The Netherlands. He obtained the ingenieur degree in chemistry in 2003. During the internship at DSM his mind was set on following a PhD program. In April 2004, he started his PhD research in the Biocatalysis and Organic Chemistry group of the Technische Universiteit Delft, The Netherlands, under the supervision of prof. Roger A. Sheldon and Dr. Fred van Rantwijk on “A Sustainable, Two-Enzyme, One-Pot Procedure for the Synthesis of Enantiomerically Pure α -Hydroxyacids”. The results of this research are described in this thesis. After finishing his PhD, the author decided to embark on a new challenge at Nissan where he currently works as a Future Technologies Research Engineer and where he paves the way for a revolution in propulsion systems. His focus lies on electric and fuel cell vehicles, on energy strategy issues, as well as on the recharging and refuelling infrastructure.

Propositions belonging to the thesis

A Sustainable, Two-Enzyme, One-Pot Procedure for the Synthesis of Enantiomerically Pure α -Hydroxy Acids

Andrzej Chmura

1. Di-isopropylether is not a green solvent.
(This thesis)
2. Bi-phasic enzymatic reactions can be a blessing for the enzymes but are a nightmare for the chemist.
3. An oxynitrilase-nitrilase double clone could be more efficient and more environmentally friendly than one using a combi-CLEA only if the reaction pH control is tightly controlled.
(Chapter 5, this thesis)
4. Enzymatic hydrocyanation at neutral or weakly basic pH is not likely to be effective at synthetically relevant concentrations.
(Von Langermann, J.; Guterl, J. K.; Pohl, M.; Wajant, H.; Kragl, U. *Bioprocess Biosyst. Eng.* **2008**, *31*, 155.)
(Kirschbaum, B.; Wilbert, G.; Effenberger, F. (Clariant Corp.) US 2002/0052523.)
5. It is not possible to draw meaningful conclusions on enzyme enantioselectivity without performing reactions in the absence of any catalyst.
(Hernandez, L.; Luna, H.; Solis, A.; Vazquez, A. *Tetrahedron: Asymmetry* **2006**, *17*, 2813-2816.)
6. Some scientists see surfactants in hydrocyanations as a problem. Others see it as an opportunity.
(Chapter 8, this thesis)
(Jackson, W. R.; Jayatilake, G. S.; Matthews, B. R.; Wilshire, C. *Aust. J. Chem.* **1988**, *41*, 203-213.)
7. Applications of α -hydroxy acids in dermatology and the nitrilase mediated synthesis of α -hydroxy acids have one thing in common: the results are clearly visible but neither dermatologists nor chemists know the mechanism of how it happens.
(Chapter 1 & 6, this thesis)
(Maddin, S. *Skin Therapy Letter* **1998** (issue 5), 3, 1-2.)
8. The electrification of the automotive propulsion power-train is not a revolution, but a return to the roots of modern mobility!
9. Changing mobility systems from one based on oil to one based on lithium does not seem to be a wise strategic move.
10. The practical application of the revolutionary silicon anode technology will have to wait until the matching cathode technology has been developed.
(Cui, L. F.; Yang, Y.; Hsu, C. M.; Cui, Y. *Nano Lett.* **2009**, *9*, 3370-3374.)

These propositions are considered opposable and defensible and as such have been approved by the supervisor Prof. Dr. R.A. Sheldon.

A Sustainable, Two-Enzyme, One-Pot Procedure for the Synthesis of Enantiomerically Pure α -Hydroxy Acids

Andrzej Chmura

1. Diisopropylether is geen groen oplosmiddel.
(Dit proefschrift)
2. Twee-fasen reacties kunnen een zegen zijn voor enzymen, maar zijn een nachtmerrie voor de chemicus.
3. Een oxynitrilase-nitrilase dubbele kloon zou efficiënter en milieuvriendelijker kunnen zijn dan een combi-CLEA, maar alleen als de pH van de reactie strikt onder controle gehouden kan worden.
(Hoofdstuk 5, dit proefschrift)
4. Enzymatische hydrocyanering bij een neutrale of zwak basische pH is hoogstwaarschijnlijk ineffectief bij synthetisch relevante concentraties.
(Von Langermann, J.; Guterl, J. K.; Pohl, M.; Wajant, H.; Kragl, U. *Bioprocess Biosyst. Eng.* **2008**, *31*, 155.)
(Kirschbaum, B.; Wilbert, G.; Effenberger, F. (Clariant Corp.) US 2002/0052523.)
5. Het is niet mogelijk om relevante conclusies te trekken over de enantioselectiviteit van een enzym zonder ook de reactie uit te voeren waarbij de katalysator afwezig is.
(Hernandez, L.; Luna, H.; Solis, A.; Vazquez, A. *Tetrahedron: Asymmetry* **2006**, *17*, 2813-2816.)
6. Sommige wetenschappers zien oppervlakte-actieve stoffen in hydrocyaneringen als een probleem. Anderen zien dit juist als een uitdaging.
(Hoofdstuk 8, dit proefschrift)
(Jackson, W. R.; Jayatilake, G. S.; Matthews, B. R.; Wilshire, C. *Aust. J. Chem.* **1988**, *41*, 203-213.)
7. De toepassingen van α -hydroxyzuren in de dermatologie enerzijds en de nitrilase gekatalyseerde synthese van α -hydroxyzuren anderzijds hebben één overeenkomst: de resultaten zijn onmiskenbaar, maar zowel dermatologen als chemici weten niet hoe het werkt.
(Hoofdstuk 1 & 6, dit proefschrift)
(Maddin, S. *Skin Therapy Letter* **1998** (issue 5), 3, 1-2.)
8. De elektrificatie van de aandrijving in auto's is geen revolutie, maar een terugkeer naar de wortels van moderne mobiliteit!
9. De verandering van vervoer gebaseerd op olie naar vervoer gebaseerd op lithium lijkt geen strategisch verstandige beslissing te zijn.
10. De praktische toepassing van de revolutionaire silicium-gebaseerde anode technologie zal moeten wachten totdat een evenarende kathode technologie is ontwikkeld.
(Cui, L. F.; Yang, Y.; Hsu, C. M.; Cui, Y. *Nano Lett.* **2009**, *9*, 3370-3374.)

Deze stellingen worden opponeerbaar en verdedigbaar geacht en zijn als zodanig goedgekeurd door de promotor prof. dr. R.A. Sheldon.