The Application of Nitrile Hydratases in Organic Synthesis

Sander van Pelt

Cover: A panorama of a Siberian hypersaline soda lake.

The Application of Nitrile Hydratases in Organic Synthesis

Proefschrift

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Contents

1

General Introduction

Sustainability, green chemistry, and biocatalysis

The World Commission for Environment and Development was founded in 1983 by the United Nations. Its function was to produce a report on the perspectives of long-term, sustainable and environmentally friendly development on a world scale by 2000 and after. Four years later this commission published its report on the future, *Our Common Future*[1], also known as the *Brundtland Report* after the Minister President of Norway and chairman of the commission at that time: Gro Harlem Brundtland. In the Brundtland report sustainable development is defined as: *Meeting the needs of the present generation without compromising the ability of future generations to meet their own needs.* This concept has become the focus of quite some attention both in industry as in society in the last two decades.

P – Prevent wastes
R – Renewable materials
O – Omit derivatisation steps
D – Degradable chemical products
U – Use of safe synthetic methods
C – Catalytic reagents
T – Temperature, Pressure ambient
I – In-Process monitoring
V – Very few auxiliary substrates
E – E-factor, maximise feed in product
L – Low toxicity of chemical products
Y – Yes, it is safe

Figure 1.1 The 12 principles of green chemistry in condensed form.[2]

Green chemistry is a concept developed to be used as a tool to accomplish sustainable development in the chemical industry and can be defined as follows: *Green chemistry efficiently utilises (preferably renewable) raw materials, eliminates waste, and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products.*[3] Instead of focusing on waste remediation, green chemistry strives for primary pollution prevention by the design of

greener processes (*benign by design*). The twelve principles of green chemistry, in condensed[2] (Figure 1.1) or elaborate[4,5] form, are the guidelines of green chemistry and should be used from exploratory research up to full scale industrial commercialisation.

The green chemistry concept is not only expressed in behavioural guidelines but also in more concrete green chemistry metrics such as the environmental factor or E factor[6], atom economy[7] (atom efficiency), process mass intensity (MI)[8], and reaction mass efficiency (RME)[9]. These metrics are all designed in an attempt to assess the "greenness" of processes, to improve process efficiency, and to reduce waste generation and energy consumption. For example, the E factor expresses the amount of waste produced compared to the amount of product obtained in kilograms (Table 1.1).[6,10-12]

Products in the fine chemical and pharmaceutical industry are usually complicated (chiral) molecules that are produced by multi-step syntheses. This is one of the reasons why the E factor increases dramatically from bulk to fine chemicals and pharmaceuticals (Table 1.1). The high E factors in the fine chemical and pharmaceutical industries are clearly in need for improvement and could be drastically reduced by using catalysts instead of the classical stoichiometric reagents.

Table 1.1 E factors in the chemical industry[6]

Industry segment	Volume (tons per annum)[a]	E factor (kg waste per kg product)
Bulk chemicals	10^4-10^6	< 1 – 5
Fine chemical industry	10^2-10^4	5 - > 50
Pharmaceutical industry	10-10^3	25 - > 100
[a.] Annual production of the product worldwide or at a single site		

Together with chemocatalysis, biocatalysis (i.e. chemical transformations catalysed by the use of a natural catalyst, such as an enzyme) is one of the greenest technologies for chemical synthesis. This is mainly due to the excellent chemical selectivity and energy efficiency of enzymatic transformations in water. Biocatalysis can possibly prevent waste generation by performing the catalytic processes with a high stereo- and regioselectivity and by preventing or limiting the use of hazardous

organic reagents and solvents. Highly energy efficient and safe processes can be designed, since enzymes can catalyse reactions at ambient temperature, pressure, and physiological pH. The atom economy can be increased and the E factor can be decreased because extensive protection and deprotection steps can be avoided. Indeed, the catalyst itself is biodegradable.

In recent years a combination of factors is causing a revolution in biocatalysis. Among these factors are the developments in large scale DNA sequencing, genome mining, protein expression, directed evolution (rational, semi-rational, or random), high-throughput screening, structural biology and metabolic engineering. In theory, these developments enable the production of any enzyme for a commercially acceptable price. Enzyme properties, such as substrate specificity, enantioselectivity, activity, stability, and pH optimum, can be modified to satisfy process demands, which in turn can lead to an increase in industrial enzyme applications. In practice, however, biocatalysis is still only reluctantly introduced in the chemical industry. Low space time yields, low productivity, as well as a high catalyst cost are commonly mentioned drawbacks of using enzymes compared to chemical catalysts or stoichiometric reagents.

Nevertheless, biocatalysis is currently used in some new “greener” routes to lower value chemicals,[13] like acrylamide, 1,5-dimethyl-2-piperidone, and nicotinamide. The predominant role for biocatalysis in the future, however, lies in the pharmaceutical sector. Several excellent reviews on the applications of biocatalysis in the pharmaceutical industry appeared in the last 2 years and they show elegant examples of enzymes used in the synthesis of precursors or active pharmaceutical ingredients for atorvastatin (Lipitor®), simvastatin (Zocor®), levetiracetam (Keppra®), β-lactam antibiotics, and others.[14-17] In the near future, biocatalysis will be fully settled as a tool for the organic (process)chemist and will be fully implemented into chemical transformations at the retrosynthetic level.

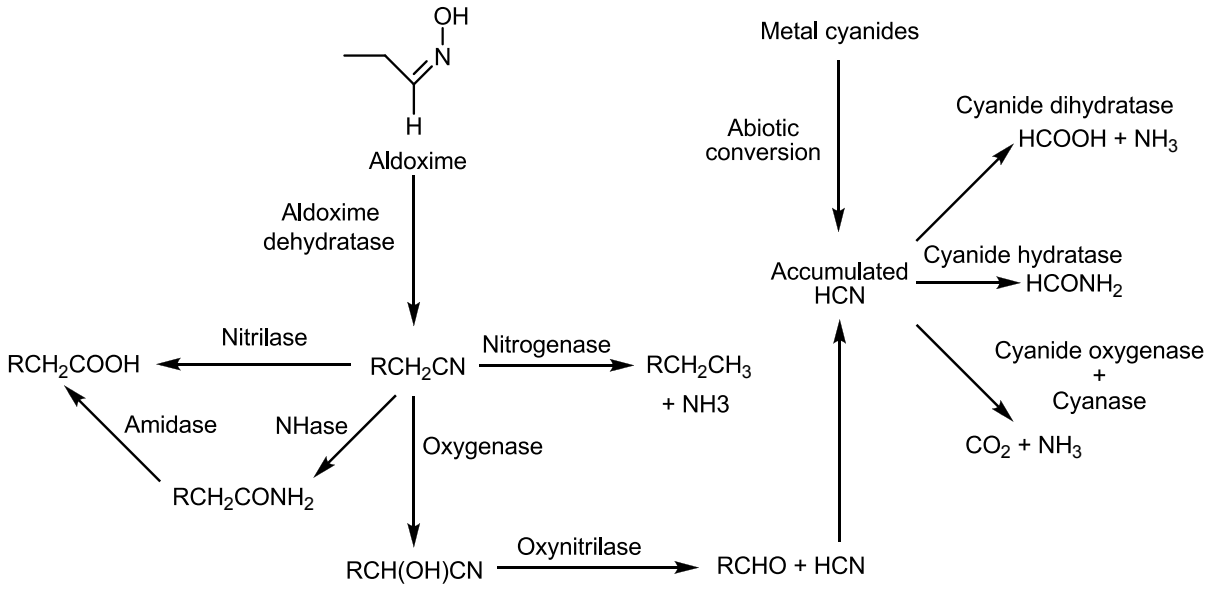
Enzymes in the CN forming and degrading pathways

Cyanide is abundant in Nature and occurs both as the inorganic hydrogen cyanide (HCN) and as organic cyanides (nitriles). A nitrile is an organic compound which has a C≡N functional group. In Nature, nitriles are mainly present as cyanoglycosides, which are amino acid derived products occurring in 2500 different species of plants. Upon tissue injury these plants process the cyanoglycosides to form a sugar, a ketone or aldehyde compound, and HCN as a self-defence mechanism (cyanogenesis).[18] Another nitrile is β-Cyano-L-alanine, which occurs widely in plants and is presumably produced in the cyanide detoxification pathway by cyanoalanine synthase from cyanide and cysteine.[19] The breakdown of glucosinolates (mustard oil glucosides) can also lead to the production of nitriles.[20] Plants can also produce nitrile compounds such as cyanolipids, ricinine, and others. In addition, simple aliphatic nitriles, such as isobutyronitrile can be produced during the anaerobic degradation of amino acids.[21]

The chemical industry also makes extensive use of various nitriles as solvents (acetonitrile), for polymer production (acrylonitrile, adiponitrile), as drug intermediates (chiral synthons), pharmaceuticals (Citalopram) and herbicides (dichlobenil, bromoxynil, ioxynil, buctril). In general, nitriles are important intermediates in the synthesis of amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones and heterocyclic compounds. Over a period of several decades, such nitriles have been widely distributed in our environment by means of industrial wastewater and residual agricultural chemicals. Most nitriles are highly toxic, mutagenic, and carcinogenic in nature.[22]

Different enzymes are responsible for the metabolism of nitriles in Nature (Scheme 1.1). The hydrolysis of nitriles is the most common pathway of nitrile metabolism and proceeds through two different enzymatic pathways.[22] In the first pathway, a nitrile hydratase (NHase, EC 4.2.1.84) catalyses the hydration of the nitrile to the corresponding amide, which can subsequently be converted by an amidase (EC 3.2.1.4) to the carboxylic acid and ammonium ion. In the second pathway a nitrilase (NLase, EC 3.5.5.1) catalyses the direct hydrolysis of a nitrile to the corresponding acid and ammonium ion. In many plants and insects nitriles can

also be oxidised by oxygenases to the cyanohydrins (α-hydroxynitriles) which can be further converted to the aldehyde or ketone and HCN by oxynitrilases (hydroxynitrile lyases, HnL, EC 4.1.2.10, EC 4.1.2.11, and EC 4.1.2.37).[18,23] Nitrogenases are capable of reducing nitrile ompounds to the corresponding hydrocarbons and ammonia[24] and the recently discovered aldoxime dehydratase (EC 4.99.1.5) transforms aldoximes to the nitrile by catalysing a dehydration reaction.[25]



Scheme 1.1 The different pathways of nitrile metabolism in Nature.[22]

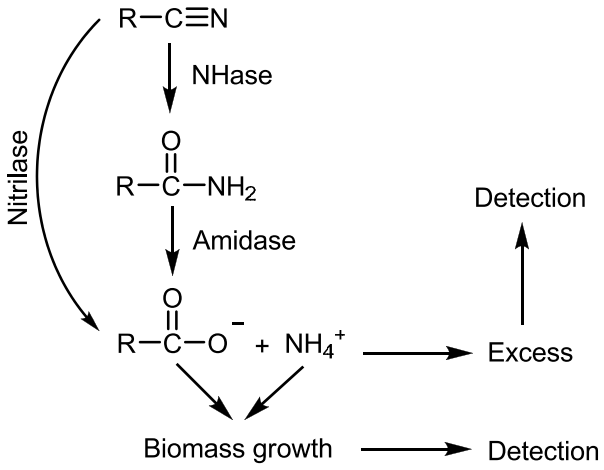
Inorganic HCN can be converted to a variety of products by the action of different enzymes. Cyanide hydratase (EC 4.2.1.66) catalyses the hydration of HCN to formamide while cyanide dihydratase (cyanidase) produces formic acid and ammonia from HCN.[26] Although not proven to be a pathway of HCN degradation in Nature, HCN can also be degraded to carbon dioxide and ammonia by the combined action of a cyanide oxygenase and a cyanase (EC 4.2.1.104).[27]

Of the variety of nitrile manipulating enzymes provided to us by Nature, so far the NHase, NLase, amidase, and HnL have found applications in industry as biocatalysts.[28,29] NHases are one class of the most successfully applied enzymes in the production of bulk chemicals as well as fine chemicals. This class of enzymes is the topic of this thesis.

Nitrile hydratases

Discovery and sources

NHases were officially discovered and named, 20 years after the discovery of NLases,[30,31] in studies on the biological degradation of toxic cyano-group containing compounds in 1980,[32] although intermediate amide formation during nitrile hydrolysis using a variety of organisms was already observed in several earlier studies.[33] NHases, in contrast to NLases, are mainly present in bacteria.[22] They are described to occur in species belonging to the phyla *Proteobacteria*, *Actinobacteria*, *Cyanobacteria* and *Firmicutes* in habitats ranging from soil,[34] via costal marine sediments[35] and deep see sediments,[36] to geothermal environments.[37] In a recent report, the first eukaryotic NHase was identified in the unicellular *Monosiga brevicollis* during a large scale screening effort for NHases in public sequence databases and metagenomic datasets. This indicates a wider phylogenetic spread of NHases than was previously assumed.[38]



Scheme 1.2 Enrichment scheme for nitrile hydrolysing bacteria.

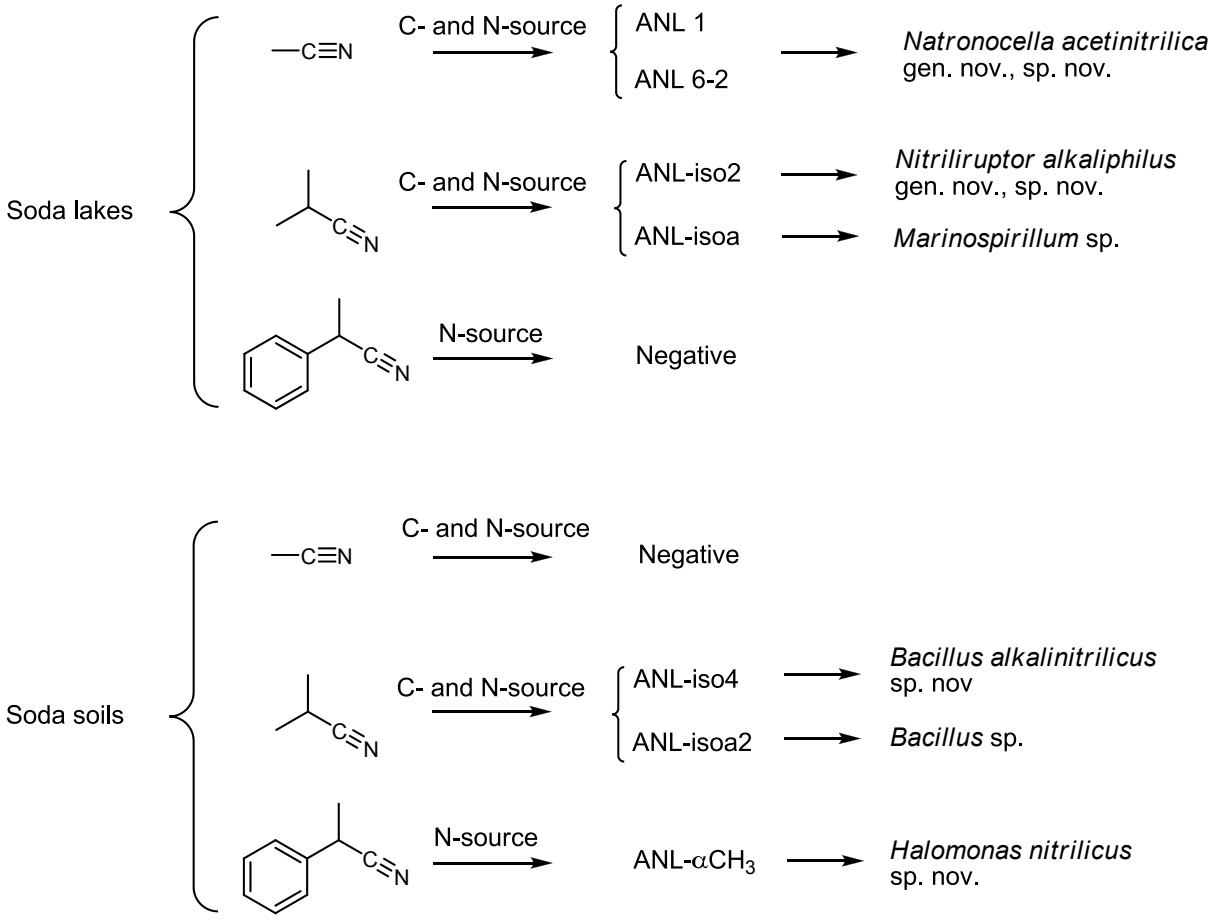
Most nitrile hydrolysing microorganisms are obtained by using a selection technique like an enrichment culture.[34] This technique is based on the repeated cultivation of mixed microbial populations of a certain source (soil, sediment, etc.) with a nitrile compound. Depending on the capability of the selected organism to

metabolise the corresponding acid, this nitrile compound can be used as the only N-source or as the only C- and N-source for the organism to grow on (Scheme 1.2). Microorganisms growing faster than other species become dominant in the culture and can be isolated. Usually, the isolates show high relative activities against their selection nitrile. Selection criteria such as temperature or pH can also be varied according to the desired properties of the whole cell biocatalyst.

From molecular ecological studies it was concluded that the microbial diversity that can be obtained by standard cultivation techniques covers only a fraction, probably less than 1 %, of the diversity present in complex environmental samples.[39] This means that > 99 % of potentially useful biocatalysts is not available. The direct cloning of environmental DNA (metagenomics) avoids the enrichment and cultivation of microorganisms and contributes significantly to the identification of new biocatalysts. The high sequence homology of all the known NHase α-subunits makes it possible to design degenerate primers, which can be used for a PCR-based method to screen metagenomic libraries from different natural habitats for the α-subunits of NHases. The full length sequences of the genes encoding the NHases can then be determined by a primer walking strategy and subsequently the enzymes can be expressed in *Escherichia coli*.[40,41] Although by the nature of this method, no NHases with a completely new structure and/or mechanism can be found, it is possible to find novel NHases with a high diversity. NHase gene sequences can also be obtained from different online genome databases such as the National Center for Biotechnology Information (NCBI).

Another method to find new organisms and enzymes is to probe extreme environments. Soda lakes and soda solonchak soils are naturally occurring saline habitats with a constant high pH of around 10 due to the high alkaline buffering capacity of dissolved sodium carbonates. These habitats harbour mostly haloalkaliphilic prokaryotic microbial communities.[42-44] At the beginning of this project there was no evidence in the literature of the possibility of nitrile biodegradation at high pH and high salt conditions. This prompted us to look at the potential for nitrile degradation in haloalkaliphilic microbial communities in mixed sediment samples from the Kulunda Steppe soda lakes (south-western Siberia, Altai region, Russia) and in mixed soil samples from soda solonchak soils (Kulunda Steppe and north-

eastern Mongolia) by using an enrichment strategy on three different nitriles (Scheme 1.3).



Scheme 1.3 The results of enrichment experiments on three different nitriles using soda lake sediments and soda soils.[45-49]

The enrichments on 2-phenylpropionitrile were only successful for soda soils when the nitrile was used as an N-source only. The isolated *Halomonas nitrilicus* contained a nitrilase and was therefore beyond the scope of this thesis.[47] The organisms isolated with acetonitrile as both the C- and N-source only showed a very low nitrile hydrating activity as well as a narrow substrate specificity, which made them less interesting from an organic chemistry point of view.[45] The enrichments on isobutyronitrile, using soda lake sediments and soda soils, resulted in two new organisms with a high level of NHase activity as well as a broad substrate specificity.

These isolated organisms and their enzymes were characterised in detail, as discussed in the Chapters 2,3, and 4.

Four NHase genes obtained from the NCBI database were successfully expressed in *E. coli* and purified using his-tag chromatography. These NHases were probed for their enantioselectivity in Chapter 4.

Crystal structure

NHases can be divided into two different groups: Fe-type NHases and Co-type NHases. The crystal structure of the Fe-type NHase, including some mutants, has been elucidated (Figure 1.2).[50-55] The α-subunit consists of a long N-terminal and a globular C-terminal domain that is best described as a four layered structure α-β-β-α. The N-terminal part of the β-subunit has a long loop of 30 residues that wraps around the α-subunit in the dimer. Residues β30-β112 fold into a helical domain that packs with the α-subunit and the C-terminal part of the β-subunit consists of a β-roll and one very short helix.

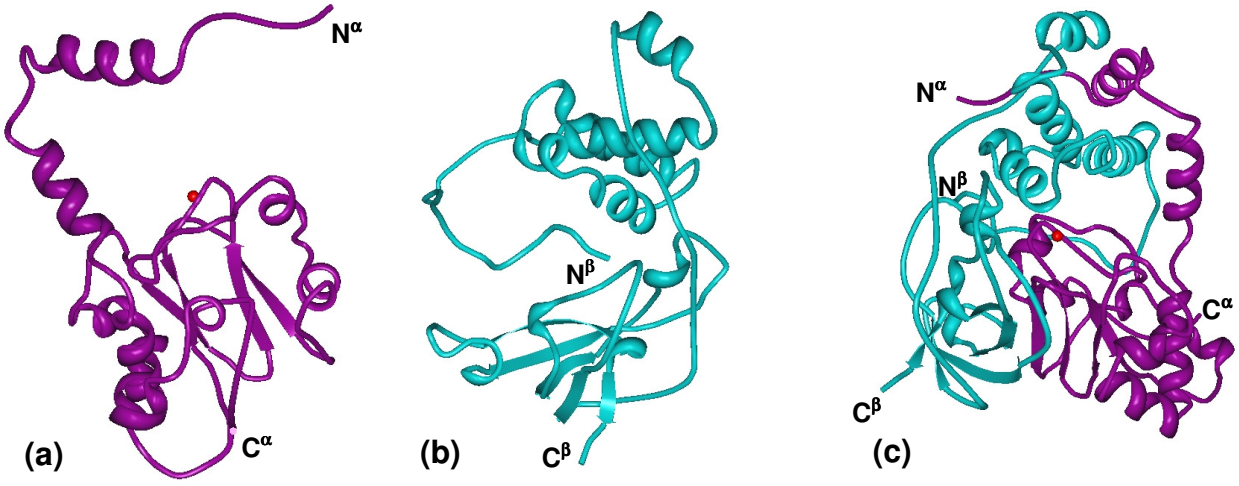


Figure 1.2 Schematic view of the α-subunit **(a)**, the β-subunit **(b)**, and the αβ-heterodimer **(c)** of the NHase from *Rhodococcus sp.* R312 (PDB ID: 1AHJ). The location of the iron centre is indicated by a red sphere. The picture was made by using PDB Protein Workshop 3.5.

The α- and β-subunits form a heterodimer which is indicated by a large subunit interface area of 3700 Å^2. There is a large open cavity between the two subunits. The metal centre is located in the interior of this cavity and is bound to the α-subunit. The

open form of the active site pocket puts only a few restraints on the size of the possible substrate, which is in agreement with the observed broad substrate specificity of NHases. There is an extensive interface of 1560 $Å^2$ between two αβ heterodimers, which may indicate that the quaternary structure of the Fe-type NHases is a tetramer. This is however not established yet, as other determination methods, such as size exclusion chromatography and gel electrophoresis point to a dimeric structure.

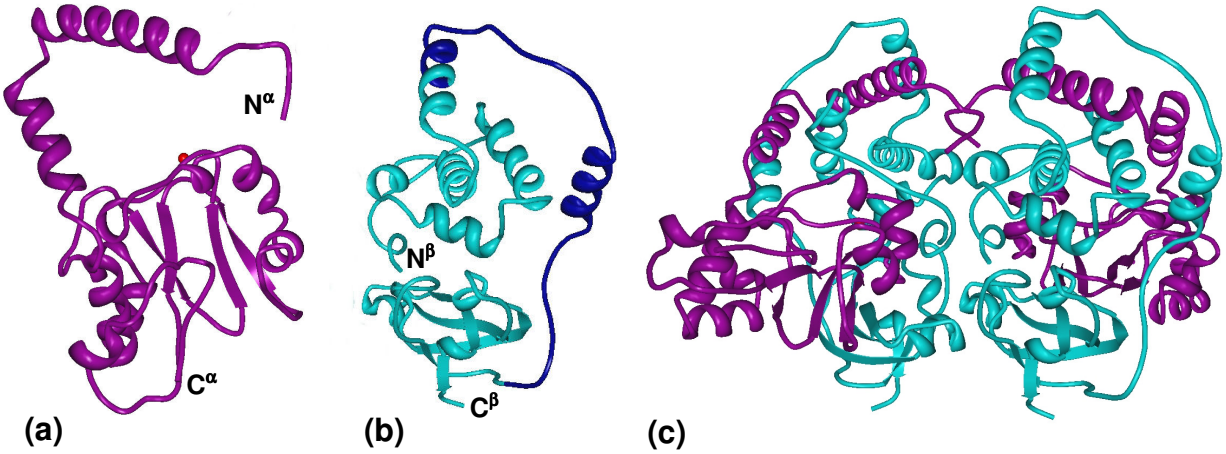


Figure 1.3 Schematic view of the α-subunit **(a)**, the β-subunit **(b)**, and the $\alpha_2\beta_2$-heterotetramer **(c)** of the NHase from *Pseudonocardia thermophila* JCM 3095 (PDB ID: 1IRE). The location of the cobalt centre is indicated by a red sphere. The residues between β95 and β138 have a dark blue colour. The picture was made by using PDB Protein Workshop 3.5.

The cobalt NHases exist as hetrotetramers (Figure 1.3).[56-58] The α-subunit of the Co-type NHase exhibits very high structural similarity with that of the Fe-type. The two β-subunits are also similar, although several structurally different parts can be detected. The residues between β95 and β138 greatly deviate from the Fe-type NHase (dark blue region in Figure 1.3). These residues interact strongly with the α-subunit and might explain the higher stability of the Co-type enzyme. Around the active site some other differences can be seen. A tryptophan residue (βTrp^{72}) conserved in the Co-type NHases corresponds to a tyrosine residue (βTyr^{76}) in the Fe-type. The difference in the direction of βTrp^{72} in the Co-type compared to βTyr^{76} in the Fe-type makes a larger space available in the substrate binding site. This

difference is assumed to be the cause of the different substrate preferences of Co-type and Fe-type NHases.[56]

The βLeu[48], βPhe[51], and βTrp[72] residues of the *Pseudonocardia thermophila* JCM3095 NHase participate in the recognition of a substrate and form a hydrophobic pocket which is thought to accommodate the alkyl chain or aromatic ring of a nitrile substrate. The differences in form and size of this hydrophobic pocket seem to produce the various substrate preferences among NHases. Instead of a βLeu[48] residue, the β-subunit of the Co-type NHase from *Bacillus smithii* SC-J05-1 contains a βPhe[52] residue that partially covers the metal centre and narrows the active site cleft. It was suggested that this structural feature contributes to the preference of this enzyme for aliphatic substrates.[58] The bulky βPhe[37] residue in the active site cleft may play a role in the enantioselectivity of some Co-type NHases.[59]

Active site structure

As mentioned previously, the metal ions in both the Fe-type and Co-type NHases are located in their α-subunits, which share a common and characteristic metal binding motif C*X*LCSC, where *X* is a serine (S) in the case of a Fe-type and a threonine (T) in the case of a Co-type NHase. Spectroscopic studies have shown that the iron ion in the active site of a Fe-type NHase is a non-heme Fe(III)[60] and that the cobalt ion in the active site of a Co-type NHase is a noncorrin Co(III).[61]

Structural analyses by X-ray crystallography for both types of NHases have revealed that they have the same geometry at the active site.[50,54,56,58,62] The active site of all known NHases is composed out of four conserved amino acid residues of the α-subunit (αCys[109], αCys[112], αSer[113], and αCys[114*]) that provide the ligands to the metal ion and two conserved residues of the β-subunit (βArg[56] and βArg[141*]) that are assumed to provide stability to the active centre through hydrogen bonding. Furthermore, they probably fine tune the electronic state of the metal centre for catalysis. Mutagenic studies have shown that these conserved arginines are

[*] Amino acid numbers refer to the Fe-type NHase of *Rhodococcus* sp. N-771

essential for catalysis.[63] The octahedrally coordinated metal ion has two deprotonated backbone amides as ligands (donated by αSer^{113} and αCys^{114}*) as well as the three sulfur atoms of the cysteine residues, of which two are posttranslationally oxidised to cysteine sulfenic (αCys^{114}-SOH) and cysteine sulfinic acid (αCys^{113}-SO_2H) (Figure 1.4). These protein ligands are consistent with earlier spectroscopic results from EPR,[60] ENDOR,[64,65] EXAFS[66,67], and resonance Raman[61,66] experiments. Fourier transform infrared spectroscopy has shown that the cysteine sulfenic and sulfinic acids are present in their deprotonated forms in the active enzyme.[68]

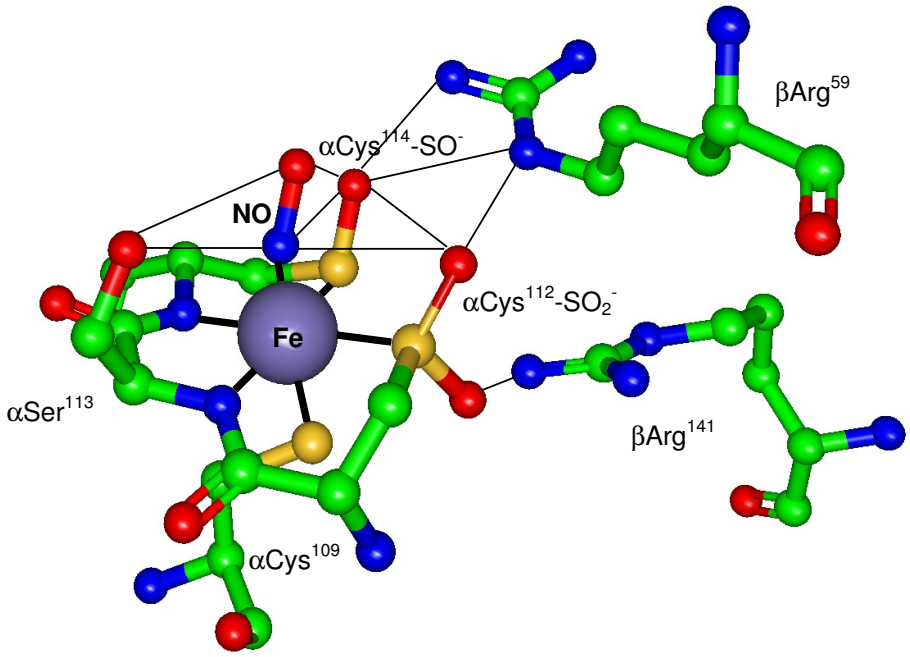


Figure 1.4 Structure of the non-heme iron active centre of the Fe-type NHase in an inactive state. The atom pairs that are within hydrogen bonding distance are linked with thin black lines. The iron ligand interactions are presented with thick black lines (PDB ID: 2AHJ). Green: carbon, yellow: sulfur, red: oxygen, blue: nitrogen, grey: iron metal centre. The picture was made by using PDB Protein Workshop 3.5.

In inactive Fe-type NHase, the sixth metal ligand is NO, which is captured in a "claw setting" of three oxygen atoms (Figure 1.4).[69] The inactivated nitrosylated Fe-type NHase can be prepared by the aerobic incubation of the microorganisms in the dark. Activity is immediately recovered upon light irradiation which causes photo-dissociation of the NO molecule.[70,71] This photo-dissociation is likely to be common

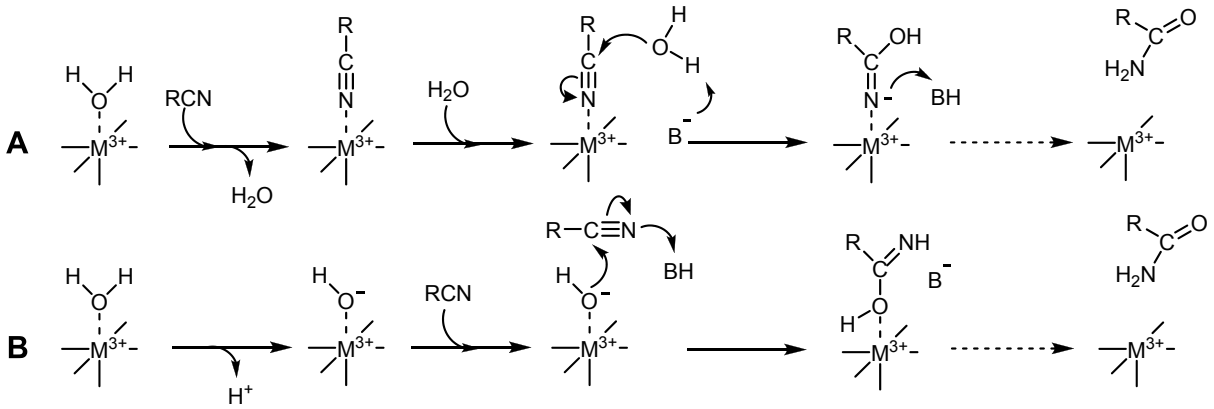
among all Fe-type NHases. Although no reports in the literature describe the production of inactivated Co-type NHases by fermentation in the absence of light, experiments on the effect of cobalt substitution on the activity of Fe-type NHases indicate that Co-type NHases are regulated by CO.[72] The sixth metal-ligand of the active NHase remains elusive. It is generally suggested to be a hydroxide ion, a water molecule, or possibly the substrate.[50,53,56,58,73]

Mechanism of catalysis

At the time of writing, there was no consensus in the literature regarding the mechanism of NHase catalysis. Two types of general reaction mechanisms for NHases have been considered repeatedly.[50,74,75] These mechanisms are described as the first-shell mechanism and the second-shell mechanism (Scheme 1.5).

First-shell mechanism

In the first-shell mechanism (Scheme 1.5, **A**) the nitrile coordinates to the metal ion directly, which could possibly act as a Lewis acid, activating the substrate towards nucleophilic attack by a water molecule.

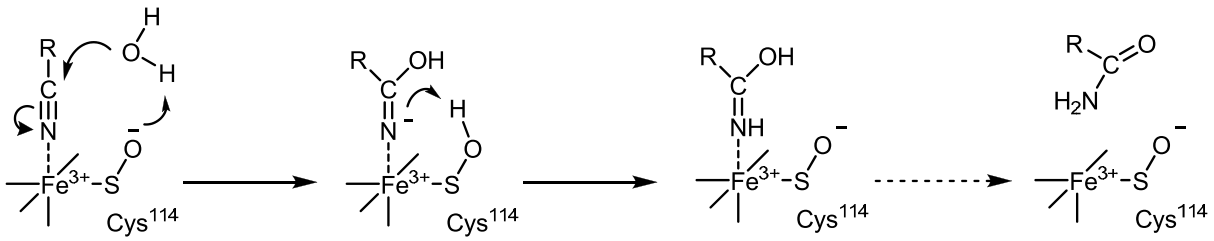


Scheme 1.5 The two possible general reaction mechanisms of NHase. The first-shell mechanism (**A**) involving direct coordination of the substrate to the metal and the second-shell mechanism (**B**) in which a metal-coordinated hydroxide ion attacks the substrate in the second shell.

The described first-shell mechanisms mainly differ in regard to the identity of the catalytic base that is thought to activate a water molecule for nucleophilic attack.[76,77]

The first-shell mechanism was proposed based on observed changes in electronic absorption and EPR spectra upon the addition of nitriles[60] as well as on synthetic model studies that have revealed that nitriles can readily exchange with low spin Fe(III) and Co(III) centres.[74,78]

One of the possible catalytic base candidates is the post-translationally modified cysteine sulfenic acid residue (Scheme 1.6). This mechanism was suggested on the basis of a theoretical investigation using the density functional theory (DFT). The model shows that the generally suggested role of the Fe(III) centre as Lewis acid, activating the substrate towards nucleophilic attack, is unlikely. Instead, the metal is suggested to provide an electrostatic stabilisation of the anionic imidate intermediate, thereby lowering the reaction barrier.[76]



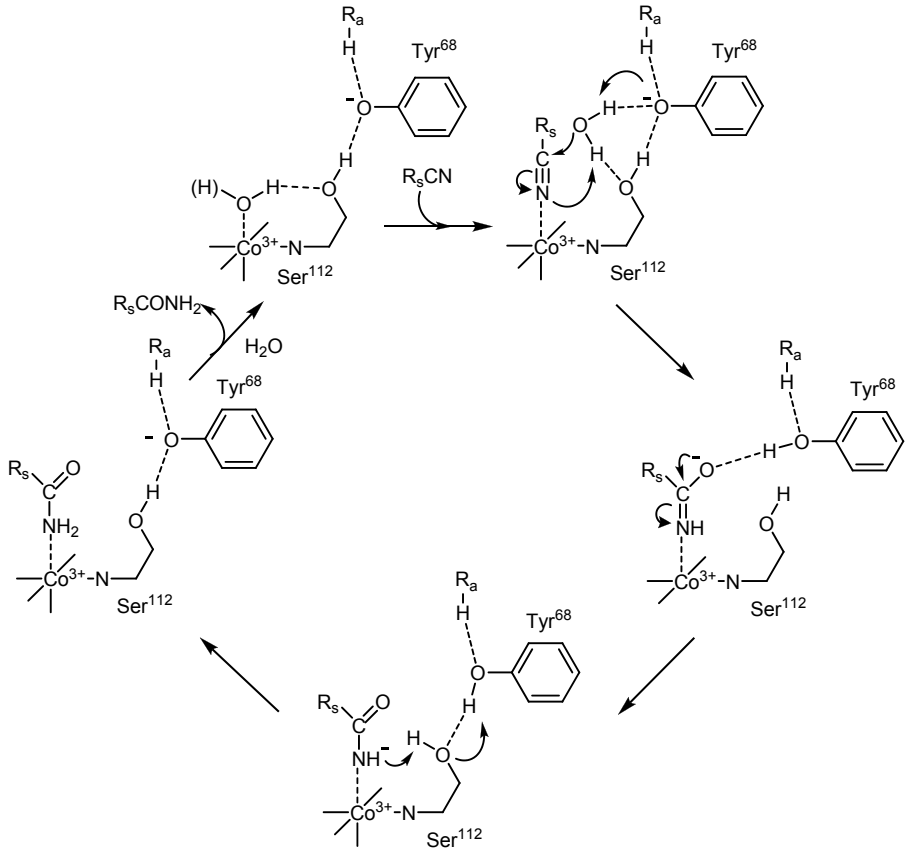
Scheme 1.6 A recently suggested variant of the first-shell reaction mechanism of NHase, where the catalytic base is the post-translationally modified cysteine sulfenic acid (αCys^{114}-SO^-) residue.[55,76,78]

This mechanism is supported by recent studies of Hashimoto et al.[55] In these studies, the conversion of isonitriles to the corresponding amines was discovered as a promiscuous activity of the Fe-type NHase from *Rhodococcus erythropolis* N-771 (*Re*NHase). The K_m for *t*BuNC was comparable to that for methacrylonitrile, whereas the k_{cat} was five orders of magnitude lower.[79] The slow reactivity of *t*BuNC as well as the photoreactivity of nitrosylated inactive *Re*NHase was used for time-resolved X-ray crystallography of *t*BuNC catalysis.[55] On the basis of these results the authors proposed a mechanism for nitrile hydration, in which the nitrile coordinates to the metal first and subsequently the nitrile carbon is attacked by a water molecule, activated by αCys^{114}-SO^-.

Involvement of αCys^{114}-SO^- in the catalytic reaction has also been suggested in previous studies using the inhibitor 2-cyano-2-propyl hydroperoxide[80] (oxidising

αCys^{114}-SO$^-$), by site directed mutants of NHases[53], by the activation of a recombinantly anaerobically reconstituted αβ complex,[81] as well as by studies on model complexes.[82]

By investigating the pH and temperature dependence of the kinetic constants k_{cat} and K_m for the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (*Pt*NHase), Mitra *et al.* suggest that once nitrile binding to the metal centre occurs, the βTyr^{68} residue will act as the catalytic base and activate a water molecule (Scheme 1.7). αSer^{112} likely functions as the general base by deprotonation of βTyr^{68}. In this mechanism, αSer^{112}, βTyr^{68}, and βTrp^{72} appear to form a catalytic triad reminiscent of those involved in non-metallodehydrogenases.[77]



Scheme 1.7 Proposed first-shell mechanism of Co-type NHase involving βTyr^{68} as catalytic base. R_s = residual group substrate, R_a = residual group active site.

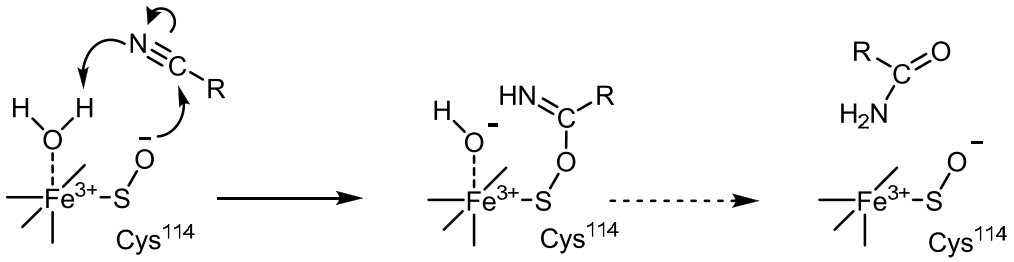
The conserved βTyr^{68} residue of the *Pt*NHase was also identified as a key residue in mutation studies.[57] A βY68F mutant had an elevated K_m value and a

significantly decreased k_{cat} value for acrylonitrile, methacrylonitrile, and benzonitrile. However, in this study, βTyr[68] is thought to be involved in the stabilisation of the imidate intermediate as well as in the stabilisation of the "claw setting" through a hydrogen bond with αSer[112].

Instead of a βTrp[72] residue, the NHase from *Comamonas testosteroni* Ni1 (*Ct*NHase) has a valine residue. The kinetic parameters as a function of pH were nearly identical, from which is concluded that the βTrp[72] residue is not required for catalysis to occur via this mechanism. However, the presence of βTrp[72] or another amino acid that is capable of hydrogen bond formation might assist in stabilising the negative charge on βTyr.[68,83]

Second-shell mechanism

In the second-shell mechanism there is no direct interaction of the substrate with the metal atom. In this case, the sixth metal ligand is a hydroxide ion, which performs a nucleophilic attack on the nitrile directly (Scheme 1.5, **B**) or through another water molecule.[50,74,75] This hydroxide ion could possibly be formed by proton transfer to αCys[114]-SO$^-$. Another second-shell mechanism was recently suggested, on the basis of DFT calculations, that involves the oxidised αCys[114]-SO$^-$ residue as the nucleophile instead of a water molecule (Scheme 1.8).[74,84]



Scheme 1.8 An alternative second-shell mechanism employing αCys[114]-SO$^-$ as nucleophile

Based on a crystal structure, Song et al. propose a second-shell mechanism where αGln[90] activates a water molecule that acts as the nucleophile.[54] Mutation studies confirm the importance of αGln[90] which is conserved in all known NHases. In these studies it is postulated, however, that αGln[90] is not directly involved in the

catalytic mechanism but is involved in the hydrogen-bond network around the catalytic centre. The k_{cat} of αQ90E and αQ90N mutants of *Rhodococcus* sp. N-771 Fe-type NHase decreased to 24% and 5 % of the wild-type respectively. The effect of these mutations on the K_m was not significant. The changes in k_{cat} could also be explained by the rearrangements in the hydrogen bond networks around the active site that are unimportant for substrate binding.[53]

DFT calculations

The barrier for NHase-mediated nitrile conversion is expected to lie in the range of 13-15 kcal/mol.[76,84,85] DFT calculations on the first-shell mechanism with αCys^{114}-SO$^-$ as catalytic base (20.2 kcal/mol) and second-shell mechanism with a metal-bound hydroxide (22.7 kcal/mol) or αCys^{114}-SO$^-$ (22.2 kcal/mol) as nucleophile have barriers which are 5-7 kcal/mol above the experimental value. The first-shell mechanism where βTyr^{68} acts as a catalytic base has a barrier for nitrile hydration of 17-21 kcal/mol, depending on the employed dielectric constant. Considering an error margin of 3-5 kcal/mol this might be the mechanism of NHase catalysis, although no real conclusions can be drawn from these calculations.[85] The previously mentioned crystal structures produced during the time-resolved X-ray crystallography of *t*BuNC catalysis show that the residues αSer^{113}, βTyr^{72}, and βTrp^{76} in *Re*NHase (αSer^{112}, βTyr^{68}, and βTrp^{72} in *Pt*NHase) were found to be unchanged during the reaction, which militates against the first-shell mechanism with βTyr^{68} as the catalytic base.[55]

Functional expression and activator proteins

Although only the amidase structural gene is required for the active heterologous expression of amidases, it was found that NHases need certain flanking sequences for successful functional expression. These flanking sequences were found to be important for functional expression in *E. coli* of both Fe- and Co-type NHases.[86-91] Some successful expressions are also described without coexpression of these flanking sequences,[92-94] which have been proposed to encode for "activators" that transport the iron or the cobalt specifically into the active site.[95] Small

proteins (12-16 kDa) are associated with the activation of Co-type NHases while the corresponding activator proteins of the Fe-type NHases have a molecular weight of 43-47 kDa.

The successful expression of the Fe-type NHases from *C. testosteroni* could also be achieved by using the *E. coli* chaperones GroES and GroEL.[96] These chaperones are members of a large group of proteins that are involved in the folding and assembly of proteins *in vivo* by the prevention of misfolding and aggregation. The addition of these chaperones was found to have no effect on NHase activity when the activator protein was coexpressed.[90] Mutagenisis on the genes encoding the NHase activator from *Rhodococcus sp.* N-771 revealed that the Fe-type activator might be participating in iron-trafficking in NHase biogenesis as an Fe-type metallochaperone.[97]

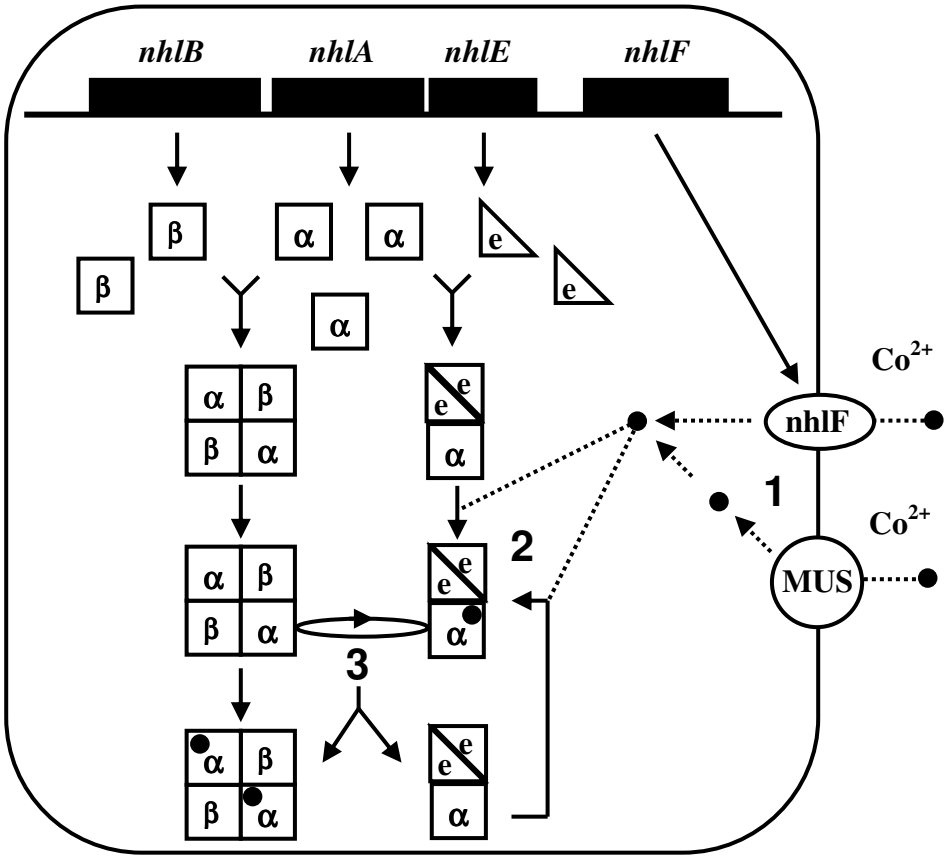


Figure 1.5 Biogenesis of the L-NHase from *R. rhodochrous* J1 using nhlE as a chaperone for "self-subunit swapping" as well as for cobalt insertion and cysteine oxidation.[98]

In two recent papers,[98,99] Zhou et al. propose a detailed mechanism for the function of the Co-type activator protein for the low molecular weight NHase (L-NHase) from *Rhodococcus rhodochrous* J1. They discovered that this activator (nhlE) acts as a chaperone for a process they named "self-subunit swapping" maturation and as a metallochaperone that is necessary for both post-translational cysteine oxidation and cobalt incorporation into the α-subunit (Figure 1.5).

In the proposed model for the biogenesis of L-NHase, a recently found cobalt transporter[100] (nhlF) and Mg^{2+} uptake systems transport Co^{2+} into the *R. rhodochrous* J1 cells (**1**). These Co^{2+} ions are then inserted into apo-αe_2, resulting in cobalt- and oxidised cysteine-containing holo-αe_2 (**2**). The formation of holo-$\alpha_2\beta_2$, active L-NHase, takes place through self-subunit swapping. The cobalt-free and non-oxidised α-subunit in apo-$\alpha_2\beta_2$ is swapped with the cobalt- and oxidised cysteine-containing α-subunit of holo-αe_2 using NhlE as a chaperone (**3**). Apo-αe_2 could be recycled for the further biogenesis of L-NHase. The driving force for the self-subunit swapping is supposed to be provided by salt-bridge formation between the deprotonated[68] oxidised cysteine residues and the two conserved arginines of the β-subunit. Dissolved oxygen was necessary for the oxidation of Co^{2+} to Co^{3+} and the oxygen atoms in the oxidised cysteine residues are proposed to be derived from water molecules. The exact mechanisms of cobalt insertion and cysteine oxidation in apo-αe_2 still remain unknown.

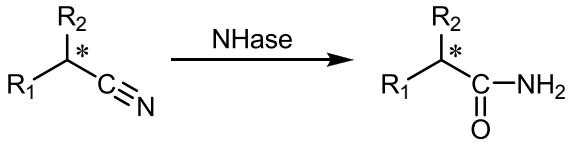
Enantioselectivity

Early experiments suggested that nitrile hydratases are relatively unspecific with respect to the chirality of the nitrile substrate and that any enantiodiscrimination occurs during the hydrolysis of the intermediate amide by an amidase.[101] However, more complex observations obtained from experiments with whole cells on arylaliphatic nitriles could only be explained by the possible existence of enantioselective NHases.[102,103]

The first enantioselective NHases were purified from *Pseudomonas putida* strain 5B[104], *Agrobacterium tumefaciens* strain d3[105,106], and *Rhodococcus equi* strain A4[107] (Table 1.2). All of these purified enzymes show mainly a preference for *(S)*-2-

arylpropionitriles and *(S)*-2-arylbutyronitriles. Optically active *(S)*-2-arylpropionamides can be useful as precursors for non-steroidal anti-inflammatory drugs like ibuprofen and naproxen, since the activities have been shown to be much higher for the *(S)*-enantiomers of these drugs. *(S)*-2-(4-chlorophenyl)-3-methylbutyramide is a precursor for the insecticide fenvalerate of which the (*S*,*S*) configuration is the most active one.

Table 1.2 Reported *E* values of > 10 for NHases in the literature



NHase source	R_1	R_2	*E* value *(selectivity)*
P. putida 5B[104,108]	4-(Cl)Ph	$(CH_3)_2CH$	~65 *(S)*[a]
A. tumefaciens[105,106,109]	Ph	CH_3	253 *(S)*[a]
	Ph	CH_3CH_2	58 *(S)*[a]
	3-(Bz)Ph	CH_3	43 *(S)*[a]
	4-(Cl)Ph	CH_3	18 *(S)*[a]
R. equi A4[107]	6-(CH_3O)naphthyl	CH_3	41 *(S)*[a]
	4-(CH_3O)Ph	CH_3	19 *(S)*[a]
P. putida 2D-11-5-1b[102]	4-(Cl)Ph	$(CH_3)_2CH$	~ 63 *(S)*[b,109]
	4-[$(CH_3)_2CHCH_2$]Ph	CH_3	~ 13 *(R)*[b,109]
P. putida 13-5S-ACN-2a[102]	4-(Cl)Ph	$(CH_3)_2CH$	~ 48 *(S)*[b,109]
Pseudomonas sp. 3L-G-1-5-1a[102]	6-(CH_3O)naphthyl	CH_3	~18 *(S)*[b,109]
Rhodococcus sp. HT 40-6[110]	Ph	OH	~ 40 *(S)*[b]
Klebsiella oxytoca[111,112]	Ph	CH_3	35 *(S)*[c,111]
	Ph	OH	19 *(S)*[c,111]
Raoultella terrigena 77.1[111]	Ph	CH_3	47 *(S)*[c]
	Ph	OH	20 *(S)*[c]
Raoultella terrigena 37.1[111]	Ph	CH_3	47*(S)*[c]
	Ph	OH	18 *(S)*[c]

[a.] Purified NHase

[b.] Whole cell catalysis

[c.] Heterologously expressed NHase

Recently, the enantioselective NHase from *Rhodococcus erythropolis* strain AJ270 was purified[113] and expressed in *E. coli*.[114] This NHase shows enantioselectivity in the hydration of several racemic *trans*- and *cis*-2-arylcyclopropanecarbonitriles. A recent patent and paper describe the enantioselectivity and heterologous expression of the NHases from *Raoultella terrigena*, *Pantoea* sp., *Brevibacterium linens*, and *Klebsiella oxytoca* (Table 1.2).[111,112] Besides the preference of these NHases for the *(S)*-enantiomer of 2-phenylpropionitrile, they also show a preference for the *(S)*-enantiomers of mandelonitrile and phenylglycine nitrile, although to a much lower extent.

BioVerdant Inc. developed a process for the production of levitiracetam in which a NHase is used to produce *(S)*-2-(2-pyrrolidon-1-yl)-butyramide from the corresponding racemic nitrile. Based on homology and docking studies, active site residues that potentially affect substrate binding and enzyme catalysis were identified and saturation mutagenesis was used to improve the enantioselectivity of the enzyme, which resulted in an *E* value of 53.[115] At the moment of writing, this is the only report in the literature of mutagenesis on a NHase to improve the enantioselectivity.

Despite an increase in reports in the literature describing enantioselective NHases in recent years, the scope of the substrates and enzymes tested is still narrow and often the data available is not sufficient to calculate the *E* values. Therefore, in Chapter 4, five purified NHases from different bacterial species are probed for their enantioselectivity on a group of 12 different chiral nitriles.

One of the ways to circumvent the lack of enantioselectivity of NHases is to combine them with an enzyme that does have good enantioselective properties. The best known example is using an amidase, which is the natural partner of the NHase, to perform a kinetic resolution on the racemic amide formed by the NHase. This cascade usually leads to *(R)*-carboxylic amides and *(S)*-carboxylic acids with good to excellent enantiomeric purities.[116] Although the combined use of an *(S)*-selective oxynitrilase and a non-selective nitrilase to produce enantiomerically pure *(S)*-mandelic acid from benzaldehyde was previously reported by our group,[117] a bienzymatic one-pot cascade of an *(R)*- or *(S)*-selective oxynitrilase with a non-selective NHase remains so far unexplored. This bienzymatic cascade offers a

possible route to the production of enantiomerically pure *(R)*- and *(S)*-α-hydroxy carboxylic amides and is discussed in Chapter 6.

Immobilisation

One of the disadvantages of NHases is that they are remarkably unstable under reaction conditions when the enzyme is used in a cell-free formulation.[113,118] This explains why most enzymatic hydration reactions are carried out using whole cells or immobilised whole cell formulations and not by using the free (purified) enzyme.[119] The preferred method of NHase immobilisation is the entrapment of whole cells with NHase activity in hydrogels such as calcium and barium alginate,[120,121] as well as in polyvinyl alcohol.[122] The technique of LentiKats, lens-shaped particles of a copolymer of polyvinyl alcohol and polyethylene glycol, was also successfully applied for the immobilisation and stabilisation of a whole cell nitrile-hydrating biocatalyst.[123] Recently also the use of carbon supports as a carrier material for whole cell NHase immobilisation was investigated.[124]

Because of the possible utilisation of products by cells, and because of the presence of other enzymes, such as amidases, esterases, alcohol dehydrogenases, and others, the use of (partially) purified cell-free NHases would be preferred over whole cell catalysis. An efficient immobilisation method for a cell-free NHase would make the application of green enzymatic hydration processes even more attractive. No information was available on the immobilisation of cell-free NHases in the literature at the beginning of this project. Therefore, in Chapter 5, the immobilisation and stabilisation of a cell-free NHase in the form of a Cross-Linked Enzyme Aggregate (CLEA) is investigated.

This method comprises the aggregation of the free enzyme from an aqueous solution using a precipitation agent, followed by cross-linking of the formed aggregates using a bifunctional cross-linking agent. Main advantages of the CLEA immobilisation technique compared to other immobilisation methods are a higher enzyme concentration per catalyst volume, which can lead to higher volumetric and space-time yields, as well as their simple and low-cost preparation.[125-127] It was

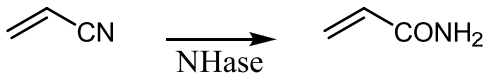
already demonstrated before that cross-linking multimeric enzymes can lead to stabilisation by the prevention of enzyme subunit dissociation.[128]

The application of NHases in industry

Acrylamide

The production of acrylamide is an example that clearly demonstrates the power of biocatalysis in industry. Acrylamide is mainly used in the production of water soluble polymers and copolymers used in flocculants, papermaking aids, thickening agents, surface coatings, and enhanced oil recovery. In 1954 American Cyanamid was the first company to produce acrylamide on a large scale. Their homogeneous approach was based on the sulphuric acid process. This process was carried out by the addition of 1 mole of acrylonitrile to a solution of 1 mole of sulfuric acid and 1 mole of water at 60-80 ^{o}C. The reaction is neutralised by the addition of ammonia, generating huge amounts of ammonium sulfate, and the formed acrylamide is purified by recrystallisation from toxic benzene.[129]

A process based on a heterogeneous Raney copper catalyst eventually replaced this stoichiometric process. The drawbacks are that this process is carried out at 120 ^{o}C and affords a product that is contaminated with traces of copper and acrylic acid as byproducts, which complicates downstream processing. Furthermore the conversion of acrylonitrile is only around 50%, which means that a significant part of the acrylonitrile has to be recycled.[129]



Scheme 1.9 Biocatalytic production of acrylamide using a NHase as catalyst.

The method of biological nitrile hydration was initially commercialised in Japan[122] where DiaNitrix* as well as Mitsui currently use the immobilised 3rd

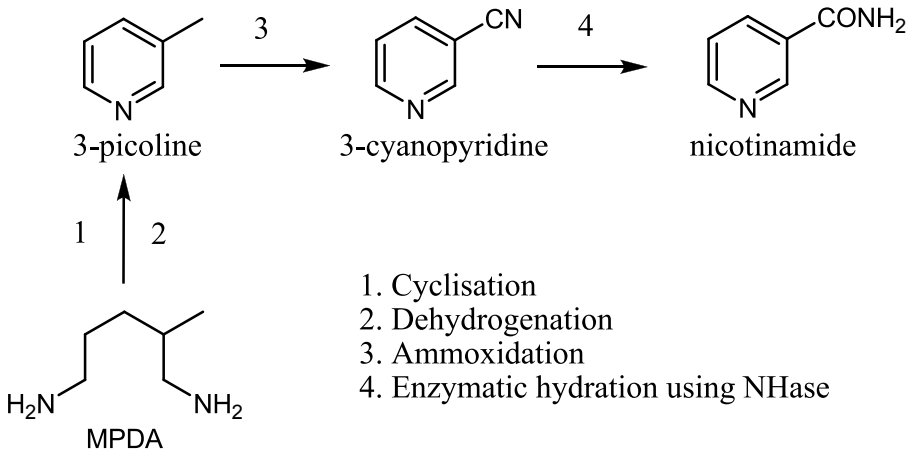
* DiaNitrix is owned by Mitsubishi Rayon and Mitsubishi Chemical Corporation

generation whole cell biocatalyst *R. rhodochrous* J1 in the combined production of approximately 120.000 ton/year of acrylamide (Scheme 1.9).[130] SNF Floerger has licensed the Mitsubishi Rayon process and has announced the production of >100.000 ton/year of acrylamide in Europe.[131] The same biological method is used in China where >30.000 ton/year of acrylamide is produced by a NHase-containing *Nocardia* sp.[132,133] It is not surprising that the enzymatic process for the hydration of acrylonitrile is slowly replacing the one which employs the heterogeneous copper catalyst, since NHases, in contrast to the Raney catalyst, catalyse the hydration reaction at low temperature, typically < 10 ^{o}C, and physiological pH in high selectivity (99.99 %) and high yield (> 99%). Acrylamide productivity of the enzymatic process is higher than 7000 grams of acrylamide per gram of dry cell weight and a time space yield of 2 kg product per liter per day can be reached.[3,134] This affords a process that is highly energy efficient, environmentally friendly, simple, and safe. The product is of such high quality that no further purification is necessary. Although research continuously takes place to find improved NHase catalysts for acrylamide production, there seems to be no candidate so far that performs better than the currently employed polyacrylamide-immobilised *R. rhodochrous* J1.[28]

Nicotinamide (vitamine B3)

A NHase is also applied by Lonza in the production process of nicotinamide (niacinamide, vitamin B3). This process is carried out in four highly selective, continuous catalytic reaction steps (Scheme 1.10) from 2-methylpentanediamine, which is a by-product of nylon-6,6 production. The last step is the hydration of 3-cyanopyridine to nicotinamide, which was originally carried out by caustic hydrolysis, resulting in copious amounts of salt waste and the formation of significant amounts of nicotinic acid as byproduct. This specific step is carried out these days by using *R. rhodochrous* J1 whole cells immobilised in polyacrylamide gel particles. Reasons for using a biocatalyst in this step are again the very mild reaction conditions, high conversion (100%) and excellent selectivity (>99.3%) of the reaction. A series of stirred-tank batch reactors is used in which a continuous feed of 3-cyanopyridine is supplied at concentrations between 10 and 20 wt% in the direction of process flow

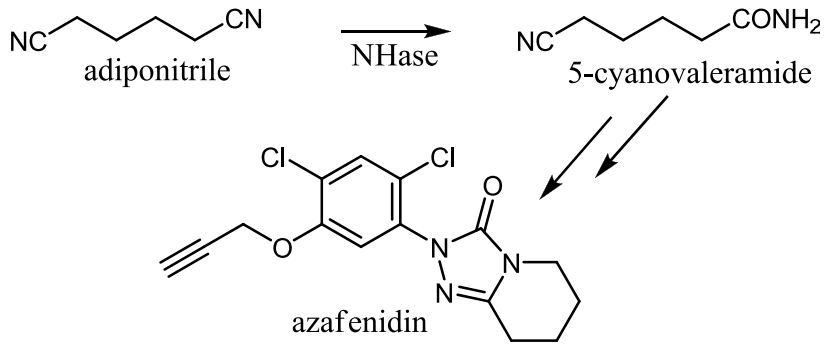
with a countercurrent feed of biocatalyst. After decolourisation of the reaction mixture and removal of the bioburden by using nanofiltration, the concentrated solution of nicotinamide is transformed into a free-flowing material of pharmaceutical quality by spray-drying without any additional purification.[135] In this way Lonza produces 11500 ton/year of nicotinamide at two different locations in Guangzhou, China, with the potential for expansion to 14500 ton/year.[136] Recent work by Lonza has focused on the development of new NHase containing organisms that do not contain a natural pigment and which have a lower K_m and a higher tolerance for 3-cyanopyridine.[28]



Scheme 1.10 Production process of nicotinamide as carried out by Lonza

5-Cyanovaleramide

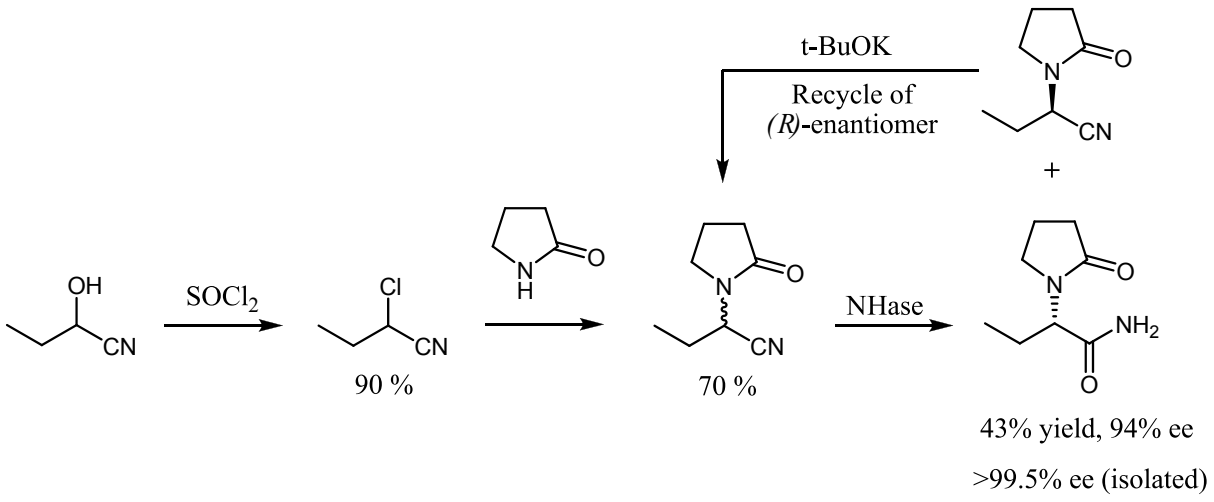
The production of 5-cyanovaleramide (5-CVAM) from adiponitrile is an elegant example of using the regioselective properties of NHases. 5-CVAM is an intermediate for the production of the herbicide azafenidin (Scheme 1.11) and is produced by DuPont using immobilised *Pseudomonas chlororaphis* B23 cells containing NHase in high conversion (97%), high yield (93%) and high selectivity (96 %). The process using a biocatalyst results in higher yields, higher catalyst productivity, less by-product formation, and generates significantly less process waste than alternative chemical methods like manganese dioxide catalysis.[137,138]



Scheme 1.11 Desymmetrisation of adiponitrile to produce 5-cyanovaleramide, a precursor of azafenidin.

Levetiracetam (Keppra®)

Keppra® is a drug used for the treatment of epilepsy with sales reaching $1.4 billion in 2007. Levetiracetam is the active pharmaceutical ingredient (API) of Keppra®. In one of the nonenzymatic production routes to this API the key step is the diastereoisomeric resolution of racemic 2-aminobutyramide using L-tartaric acid affording the desired *(S)*-2-aminobutyramide, which was subsequently reacted with 4-chlorobutanoate to give levetiracetam. This resolution has a yield of only 35% and it is difficult to recycle the undesired *(R)*-enantiomer which results in a high E factor. Additionally a hazardous alkylating agent was used in the final synthesis step.[14]



Scheme 1.12 Chemoenzymatic route for the synthesis of levetiracetam[115]

Bioverdant recently disclosed a chemoenzymatic process for the preparation of levetiracetam.[115] The most important step in this process is the NHase catalysed kinetic resolution of racemic 2-(2-pyrrolidon-1-yl)-butyronitrile (Scheme 1.12), which is prepared by the N-alkylation of 2-pyrrolidinone with racemic 2-chlorobutyronitrile. The resolution is carried out with a substrate loading of 100 $g \cdot L^{-1}$ and a target enzyme loading of ~ 4% (wt/wt). Through semi-rational engineering, a NHase mutant was obtained with increased enantioselectivity for the substrate (E = 53). The biocatalytic process is more atom efficient because the unwanted *(R)*-enantiomer could be efficiently recycled. The hazardous alkylation chemistry was circumvented by a much more friendly S_N2 reaction using an inexpensive pyrrolidinone.[14]

Other interesting future applications

Recent reviews give an excellent overview of other synthetic applications of NHases.[28,116,119] Promising are those applications that are impossible or difficult to perform using “conventional” chemical methods. These applications make use of the regioselective, chemoselective and enantioselective properties of NHases. As was already demonstrated in industry with the production of 5-CVAM, the desymmetrisation of di- or trinitriles can be a very important synthetic step. When acid or base catalysed hydration is applied for the desymmetrisation of di- or trinitriles it is necessary to stop the reaction at very low conversions, usually around 20%, to prevent the hydration of more than one nitrile group. Using a NHase possibly affords cyanocarboxylic amides in one step and in quantitative yield.

NHases are also good candidates for the chemoselective hydration of nitriles containing other reactive functionalities like ester, hydroxyl and amino groups. Since chemical hydration usually requires harsh reaction conditions, the mild hydration using NHases reduces byproduct formation caused by side reactions of other functionalities or by further hydrolysis of the amide to the carboxylic acid.

Enantioselective NHases could be used to produce enantiomerically pure amides from racemic nitriles in a kinetic resolution reaction. The dynamic kinetic resolution of nitriles that can be racemised in-situ, like α-hydroxy nitriles, could lead to enantiomerically pure amides in 100% yield. Asymmetric synthesis of α-amino or

α-hydroxy amides could also be possible when a NHase is combined with an in-situ Strecker or hydrocyanation reaction.

NHases or NHases and amidases can be used for the enzymatic treatment of polyacrylonitrile polymers. It was shown that these enzymes can be used to hydrate or hydrolyse the nitrile groups on the surface of fibres. This enzymatic treatment produced more hydrophilic fibres, imbued them with improved antistatic properties, and made them more susceptible to acidic dyes to which they were otherwise inert.[139,140]

Nitrile compounds are used extensively in the manufacture of a variety of polymers, (agro)chemicals, and pharmaceuticals. Since most of these compounds are toxic, carcinogenic, and mutagenic in nature, there is a great need to control their release into the environment. Microorganisms containing nitrile degrading enzymes, like NHases, can be used in treating nitrile contaminated wastewater streams for example in the form of an adapted activated sludge consortium.[141] However, because of the hostile environmental conditions, many wastewaters are problematic for biological degradation. Research is currently carried out to identify microorganisms that are not only effective candidates for the degradation of nitrile compounds but are also able to tolerate the additional environmental stress and the competition with indigenous microbial populations within the environment in which they will be operating. The use of new immobilisation methods for whole cell or cell-free systems might also offer an opportunity to treat more concentrated wastestreams.[142]

Other nitrile hydrating catalysts

Amide-producing nitrilases

Although the name nitrilase implies that the enzyme in question is a nitrile hydrolase that forms carboxylic acids and ammonia from nitriles, the formation of modest amounts of amides by this class of enzymes has been described in reports in literature dating as far back as the 1960s.[143] This side activity was largely neglected during several decades. Amide formation could have been ascribed to the presence of a NHase pollution or could have gone unnoticed by the presence of an amidase and because the catalytic activity was determined by ammonia release. In more recent reports, nitrilase substrates that are converted with high amide : acid ratios by certain purified nitrilases are described such as: 3-nitroacrylonitrile (95:5), 3-cyanoacrylonitrile (93:7), α-fluoroarylacetonitriles (~85:15),[144] *(R,S)*-2-chloro-2-phenylacetonitrile (89:11), and *(R)*-mandelonitrile (89:11).[145] The formation of the two alternative products is due to the competition of two competing branches of the nitrilase reaction mechanism and is dependent on the electronic and steric effects of the substrate.[145]

Research is currently carried out to improve the substrate specificity and enantioselectivity of nitrilases on a molecular basis by identifying the amino acid residues responsible for these characteristics.[146] Finding the nitrilase/NHase switch by rational mutagenisis or directed evolution will contribute to the prevention of amide side-product formation or might result in the complete conversion of nitrilases into (enantioselective) amide-forming NHases and therefore increase the catalytic usefulness of this group of enzymes.

Transition metal catalysis

A host of predominantly homogeneous organometallic and coordination complexes of early and late transition metals have been shown to catalyse the conversion of nitriles to amides.[147,148] In general these complexes activate the nitrile

by removing electron density from the nitrile carbon. The activated nitrile is then more susceptible to intramolecular or intermolecular nucleophilic attack by a water molecule or hydroxide ion. The metal centre lowers the activation barrier to hydration of the nitrile relative to hydrolysis of the amide and in many cases further hydrolysis of the amide to the carboxylic acid is not observed or highly suppressed. Cadierno et al. recently developed a hydration protocol in pure water under neutral conditions with high activity based on ruthenium.[149] However, these homogeneous methods still suffer various drawbacks. Especially the difficulty in separation of the product and expensive metal catalyst from the reaction mixture as well the use of inert atmosphere for the handling of air-sensitive metal catalysts are problematic.

Several heterogeneous systems have also been reported, like ruthenium hydroxide supported on alumina[150] and nanoparticle supported silver and ruthenium catalysts.[151,152] However, turnover numbers of these protocols are still very low, recycling of the catalyst is difficult, and elevated temperatures are often needed.

No reports in literature describe enantioselective nitrile hydration by metal complexes and although numerous synthetic analogues of Fe-type and Co-type NHases have been prepared, the highest activities obtained with these complexes are several turnovers per hour.[74]

Scope of this thesis

The scope of this thesis was to further investigate and to broaden the application of NHases in organic chemistry. Since no NHases were commercially available at the beginning of this study, soda lake sediments and soda solonchak soils from Russia and Mongolia were used in an attempt to isolate new nitrile degrading organisms as well as new nitrile converting enzymes by using the enrichment technique on different nitriles. The results of the enrichments on isobutyronitrile using these natural samples are discussed in Chapter 2. The highly active NHase-containing haloalkaliphilic bacterium *Nitriliruptor alkaliphilus* was the most active and stable of the isolated organisms. The characteristics of this whole cell biocatalyst as well as a preliminary characterisation and the purification of its NHase are reported in Chapter 3. In cooperation with NZomics, a pioneer in the commercialisation of recombinantly expressed and cell-free purified NHases, a small library of 5 purified NHases, including the one from *Nitriliruptor alkaliphilus,* were probed for their enantioselectivity in the hydration of chiral nitriles in Chapter 4. By means of producing cross-linked enzyme aggregates (CLEAs) of the relatively unstable cell-free NHase from *Nitriliruptor alkaliphilus*, an immobilised nitrile hydrating biocatalyst was obtained with a highly increased stability in Chapter 5. These CLEAs combined with CLEAs of the HnL from *Manihot esculenta* were subsequently used in a bienzymatic one-pot cascade reaction to produce enantiomerically enriched α-hydroxyamides from the corresponding aldehydes and HCN.

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2

Microbial Isobutyronitrile Utilisation under Haloalkaline Conditions

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Abstract

The utilisation of isobutyronitrile (iBN) as a C- and N-source under haloalkaline conditions by microbial communities from soda lake sediments and soda soils was studied. In both cases, a consortium consisting of two different bacterial species capable of the complete degradation and utilisation of iBN at pH 10 was selected. The soda lake sediment consortium consisted of the new actinobacterium *Nitriliruptor alkaliphilus* and a gammaproteobacterium from the genus *Marinospirillum*. The former was capable of a fast hydration of aliphatic nitriles to the corresponding amides using a nitrile hydratase (NHase) and a much slower further hydrolysis of the amides to the carboxylic acids using an amidase. Its partner cannot hydrolyse nitriles but grew rapidly on amides and carboxylic acids, thus acting as a scavenger of the products released by the actinobacterium. The soda soil consortium consisted of two *Bacillus* species (RNA group 1). One of them, the new *Bacillus* species *Bacillus alkalinitrilicus,* initiated nitrile hydrolysis, and the other utilised the hydrolysis products isobutyramide (iBA) and isobutyrate (iB). In contrast to the actinobacterium, the nitrile hydrolysing soil *Bacillus* grew rapidly with the hydrolysis products, but it was dependent on vitamins most probably supplied by its product utilising partner. All four bacterial strains isolated were moderately salt-tolerant alkaliphiles with a pH range for growth from pH 7.0 to 8.5 up to 10.3 to 10.5. However, both their NHase and amidase activities had a near neutral pH optimum, indicating an intracellular localisation of these enzymes. Despite this fact, the study demonstrated a possibility of whole cell biocatalytic hydrolysis of various nitriles at haloalkaline conditions.

Introduction

Nitriles are organic compounds containing a -C≡N (nitrile) bond. They are industrially produced as intermediates and building blocks in organic synthesis as well as organic solvents. There are also a few examples of naturally occurring nitriles formed by cyanogenic plants from cyanide.[1] In addition, simple aliphatic nitriles, such as isobutyronitrile (iBN), can be produced during the anaerobic degradation of amino acids[2].

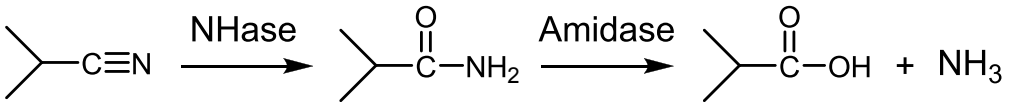
Most of the nitriles are hydrophobic and toxic compounds that are difficult to degrade. Therefore, the environmental role of the enzymatic conversion of nitriles is very important. Two different enzymatic mechanisms that catalyse the conversion of nitriles to their corresponding carboxylic acids are known. In the first mechanism the metalloenzyme NHase hydrates a wide range of aliphatic, arylaliphatic, and aromatic nitriles to their corresponding amides. These amides can then be further converted into carboxylic acids and ammonium by amidases. In the second mechanism the enzyme family of nitrilases performs a single-step hydrolysis of nitriles, mostly aromatic, into acids and ammonium, although the production of amides as by-products was reported in some cases.[3-5] The organisms producing NHases usually also produce amidases.[6] In the case of an organism with a weak amidase activity, an association with an amide specialising partner can be very efficient in order to achieve complete nitrile biodegradation.[7]

The microorganisms possessing these enzymes are valuable biocatalysts and can be used either in (enantioselective) organic synthesis or in environmental biotechnology.[7-11] This stimulated the search for active producers of nitrile-hydrolysing enzymes,[12] as well as the screening of environmental DNA and whole-genome sequences for the genes encoding new nitrile degrading enzymes.[1] Currently many strains, mostly bacterial but also several fungal, are known as active producers of nitrile hydrolysing enzymes. The best-studied group among them, producing extremely active NHases and nitrilases, belongs to the genus *Rhodococcus* in the actinobacteria.[6,13] So far, all known nitrile degrading microorganisms are neutrophilic, i.e., they grow optimally at neutral pH values.

Soda lakes and soda solonchak soils are naturally occurring saline habitats with a constant high pH of around 10 due to the high alkaline buffering capacity of dissolved sodium carbonates. These habitats harbour mostly haloalkaliphilic prokaryotic microbial communities.[14-16] Recently, we have described the first example of a bacterium, *Natronocella acetinitrilica*, isolated from soda lake sediments capable of growth with aceto- and propionitrile as carbon, energy, and nitrogen sources under haloalkaline conditions.[17]

Enzymatic nitrile hydrolysis at highly alkaline conditions might have certain advantages. Particularly when cyanide is involved in the reaction process. For example, the well-known Strecker reaction could be coupled with enzymatic α-aminonitrile hydrolysis to (enantioselectively) produce α-aminoamides and α-amino acids.[18]

In this chapter, the possibility of the degradation of more complex nitrile molecules by haloalkaliphilic bacteria from soda habitats is described. The results demonstrated that iBN can be efficiently utilised as a carbon, energy, and nitrogen source at high pH by the concerted action of at least two different bacterial species producing NHase and amidase (Scheme 2.1).



Scheme 2.1: iBN hydrolysis by a NHase/amidase system through isobutyramide (iBA) to isobutyrate (iB).

Results and Discussion

Soda lake sediment coculture

In a soda lake sediment primary enrichment culture, iBN degradation was noticeable after two weeks of incubation. Several stabilising 1:100 transfers resulted in a persistent coculture which could not be separated by the serial dilution technique. It consisted of long, motile spirilla as a dominant morphotype and short, nonmotile rods as a minor component (Figure 2.1a).

Since colony formation was not observed on solid medium with iBN, attempts were made to separate the consortium by dilution to extinction under different conditions. When the iBN concentration was gradually increased from 2 to 10 mM, the spirilla were markedly inhibited. This eventually allowed the purification of the iBN degrading rod-shaped component, designated strain ANL-iso2 (NCCB 100119, DSM 45188, UNIQEM U239), in a pure culture (Figure 2.1b). In a pure culture, ANL-iso2 was able to form flat, spreading microcolonies on iBN agar after a month of incubation. When iBN was replaced by iBA or iB, the spirilla grew much faster, and they also formed colonies on iBA agar, allowing the isolation of a pure culture designated strain ANL-isoa (Figure 2.1c). This bacterium was not able to grow with iBN.

16S rRNA gene sequence analysis showed that the nitrile degrading strain ANL-iso2 represents a new lineage in the *Actinobacteria*, mostly consisting of uncultured representatives, with maximum sequence similarities of 94 to 95 % to clones from the soda lake Mono Lake. Its partner, strain ANL-isoa, is a new species within the genus *Marinospirillum* in the *Gammaproteobacteria* with 95 % sequence similarity to its closest relative *M. alkaliphilum*.

Further investigation of the isolates confirmed that the actinobacterium was responsible for the primary hydrolysis of iBN, while the *Marinospirillum* sp. acted as a scavenger of the products, although ANL-iso2 was able to utilise the intermediates by itself. Therefore, the consortium can be characterised as commensalic, where only one organism obligately depends on the other.

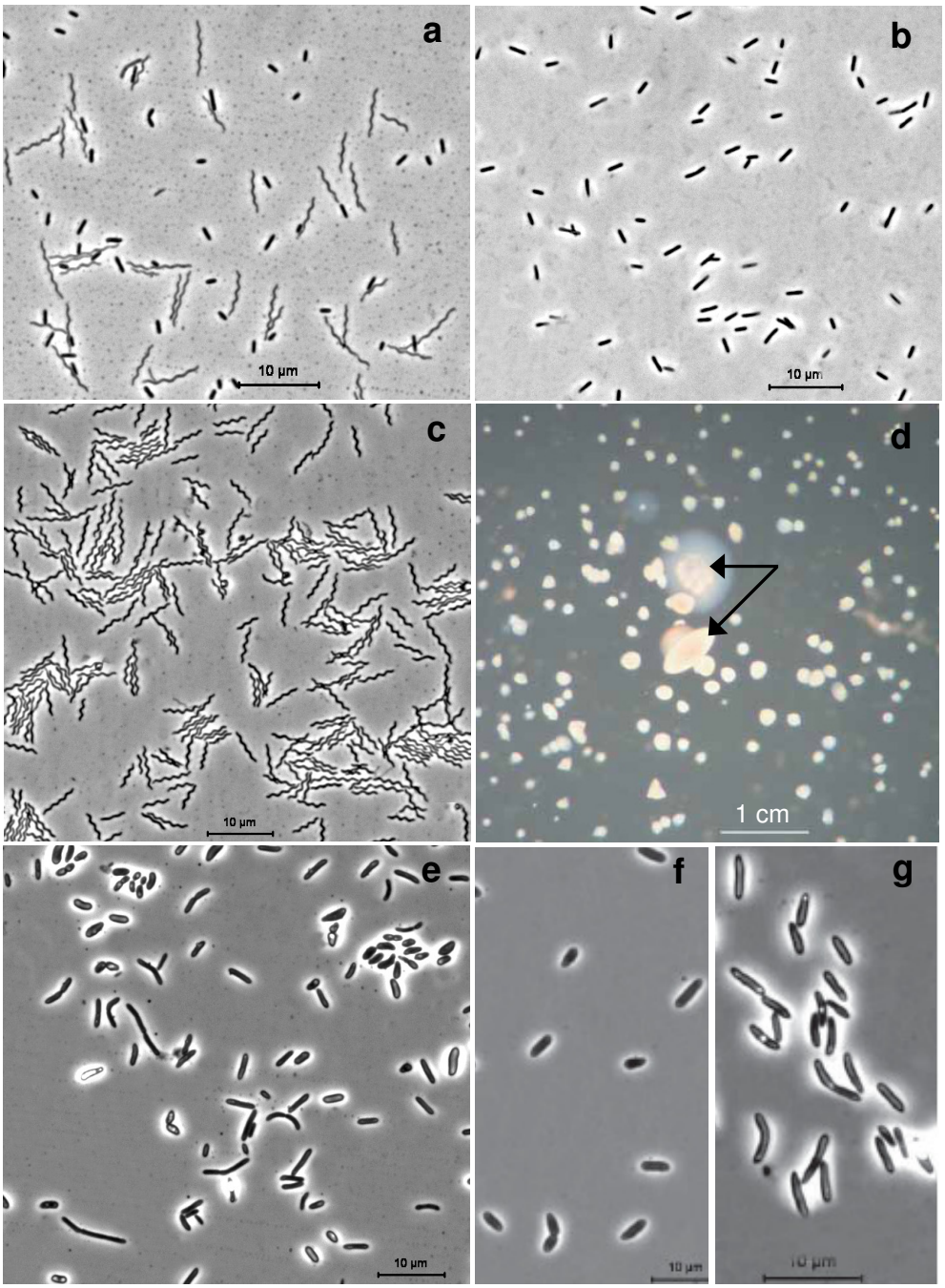


Figure 2.1 Morphology of the haloalkaliphilic bacteria involved in iBN utilisation enriched from soda lake sediments (**a-c**) and soda solonchak soils (**d-g**). (**a**) The consortium enriched from soda lakes. (**b**) The nitrile ultilising strain ANL-iso2 and (**c**) its amide utilising partner strain ANL-isoa. (**d**) Colony clusters formed by the soda soil consortium on solid medium with iBN. The nitrile utilising member (ANL-iso4) is represented by a few large colonies in the centre surrounded by numerous smaller colonies of its partner (ANL-isoa2). (**e**) The consortium, enriched from soda soils. (**f**) The nitrile utilising member of the soil consortium strain ANL-iso4 and (**g**) its amide utilising partner strain ANL-isoa2.

Table 2.1 Comparison of the growth kinetics of members of the haloalkaliphilic iBN utilising cocultures

Substrate	Specific growth rate (h^{-1}) of[a]:			
	Soda lake sediment coculture strain		Soda soil coculture strain	
	ANL-iso2	ANL-isoa	ANL-iso4	ANL-isoa2
iBN	0.035	0	0.17	0
Propionitrile	0.035[b]	0	0.14[c]	0
Butyronitrile	0.030	0	0.28	0
Valeronitrile	0.030	0	0.23	0
Capronitrile	0.030	0	0.29	0
iBA	0.044	0.15	0.19	0.13
iB	0.045	0.25	0.16	0.33
Butyrate	0.055	0.22	0.20	0.22
Acetate	0.050[b]	0.26	0.12[d]	0.66

[a.] Cocultures were grown at pH 10, 0.6 M Na^+. Values are the averages of the results of two experiments.

[b.] Growth started after a lag phase of 100 h.

[c.] Growth started after a lag phase of 200 h.

[d.] Growth started after a lag phase of 40 h.

The key to understanding why a coculture was selected instead of a monoculture completely degrading iBN, lies in the growth kinetic differences between the two organisms. The actinobacterium ANL-iso2 grew with propionitrile (C_3), butyronitrile (C_4), valeronitrile (C_5), and capronitrile (C_6) in addition to iBN as carbon, energy, and nitrogen sources (Table 2.1). Two phases of growth could clearly be distinguished. The first rapid phase of nitrile hydrolysis to the corresponding amide with little biomass growth was followed by the second, much longer, phase of biomass growth with amide and acid utilisation (Figure 2.2). The highly disproportionate rates of catabolism of iBN and the further utilisation of the products by the actinobacterium allowed the *Marinospirillum*, a scavenger growing 3-6 times faster on the iBN degradation products (Table 2.1), to efficiently pair with the slowly growing nitrile hydrolysing partner (a provider). Being unable to hydrolyse the nitrile itself, ANL-isoa is obligately dependent on ANL-iso2. Thus, the presence of two organisms with different specialisations allowed for a more efficient iBN utilisation. The fact that the *Marinospirillum* sp. has been selected as a scavenger for iBA might

not be accidental, assuming the potential of this particular group to utilise various organic nitrogen compounds originated from anaerobic protein degradation.[19]

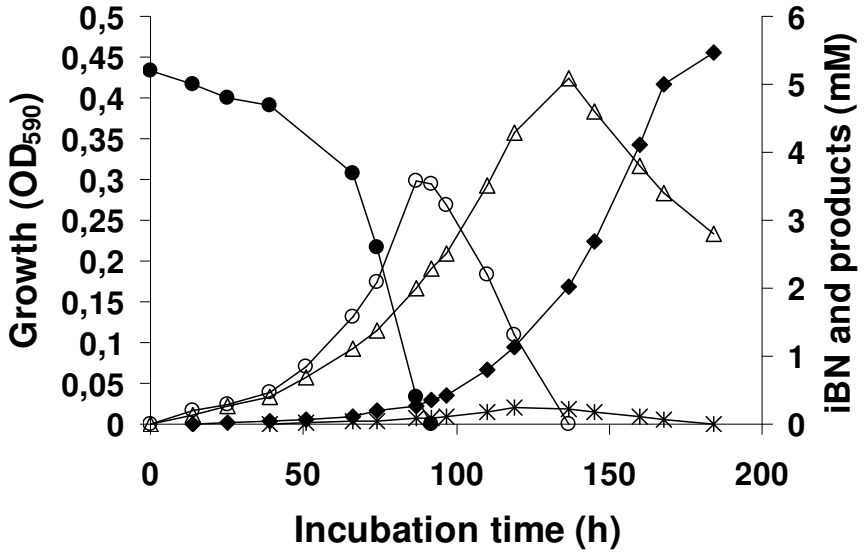


Figure 2.2 Growth and product formation with iBN as carbon, energy, and nitrogen source in batch cultures of actinobacterium strain ANL-iso2, iBN (●), iBA (○), NH_3 (Δ), iB (*) , biomass (♦) The means of the results of two experiments with deviations of < 10 % are shown.

Soda soil coculture

The soda soil enrichment culture developed much faster than the soda lake sediment enrichment culture, consuming 4 mM iBN within a week. Despite the development of quite a dense culture, serial dilutions of the 4 mM iBN medium that still contained at least two different spore-forming cell phenotypes (Figure 2.1e) were only positive up to a 10^{-8} dilution.

The phenotype with longer cells was separated from the coculture on solid medium with iBA, and the strain was designated ANL-isoa2 (Figure 2.1g). It was not able to grow with iBN. The second organism, apparently the iBN degrader, was difficult to isolate, since it was obligately dependent on its partner, in contrast to the iBN degrading actinobacterium. Eventually colony formation in a somewhat unusual fashion with iBN was observed using the agar-shake technique. Instead of a homogenous distribution, patches of growth were formed. Each patch consisted of

numerous small colonies (belonging to strain ANL-isoa2) clustered around one or two much bigger central colonies (Figure 2.1d). The latter belonged to the iBN degrading organism, as was proven by transferring those colonies into liquid medium with iBN. It was designated strain ANL-iso4 (NCCB 100120, UNIQEM U240) (Figure 2.1f). It grew relatively fast with iBN, iBA, and iB as carbon and energy sources, but only in the presence of yeast extract (50 mg L^{-1}) or a vitamin mixture. Further experiments proved that a combination of thiamine and biotin was sufficient to replace the yeast extract. The primary hydrolysis of iBN was still possible without vitamins, but the growth of biomass was impaired. The final biomass yield on iBN, iBA, and iB without vitamins was four to five times lower than with the same substrates in the presence of vitamins (Table 2.2).

Artificial reunification of the consortium from two pure cultures restored the complete degradation of iBN and the level of biomass production. From this it can be concluded that the iBN utilising coculture selected from soda soils belonged to a true mutualistic type of consortium, where both members were obligately dependent on each other. The nitrile hydrolysing member, strain ANL-iso4, depended on vitamins supplied by the second organism, which in turn, utilised the products of iBN hydrolysis. Despite the fact of the very efficient coupling of iBN hydrolysis and resulting amide utilisation by ANL-iso4, the amide specialised members of both soda lake and soda soil consortia, i.e. ANL-isoa and ANL-isoa2, were able to grow together with ANL-iso4 using iBN as substrate. This might indicate, that these bacteria have a high affinity for the amide and acid (probably within a micromolar range), allowing them to efficiently compete with the ANL-iso4 for the products of nitrile hydrolysis.

According to 16S rRNA gene sequence analysis both strains belonged to the genus *Bacillus* (RNA group 1). *Bacillus* ANL-iso4 forms a separate cluster with an undescribed soil *Bacillus* sp. BA299 (98 % similarity) and with the haloalkaliphilic, anaerobic *Bacillus arseniciselenatis* from Mono Lake, which was its closest culturable relative (95 % sequence similarity). *Bacillus* ANL-isoa2 has a 95 % sequence similarity to *Bacillus arseniciselenatis* and bacillus ANL-iso4. Apparently, both strains represent new *Bacillus* species.

Table 2.2 Influence of vitamin supplementation and a microbial partner on the efficiency of the growth of nitrile degrading soil *Bacillus* strain ANL-iso4 at pH 10[a]

Substrate (5 mM)	Supplementation[b] or microbial partner	Final biomass (OD_{600})[c]	Final concn iBN (mM)	Maximum concn iBA (mM)	Maximum concn iB (mM)
iBN	Without vitamins	0.10	0	0	0.30
	With vitamins	0.59	0	0	0
	Bacillus ANL-isoa2	0.55	0	0	0
iBA	Without vitamins	0.10		NA	NA
	With vitamins	0.59		NA	NA
iB	Without vitamins	0.10			NA
	With vitamins	0.63			NA

[a.] The values are the averages of the results of two experiments with deviations of < 10 %. NA, not analysed.

[b.] Vitamins, 10 µg L^{-1} of biotin and thiamine.

[c.] OD_{600}, optical density at 600 nm.

A comparison of the growth characteristics of the two soda soil *Bacillus* isolates showed some similarities to but also differences from the soda lake sediment isolates. Although the nitrile degrading member, strain ANL-iso4, was able to grow similarly to ANL-iso2 with a range of aliphatic nitriles (Table 2.1), its growth kinetics with nitriles were quite different (Figure 2.3). Not only did it grow several times faster, but the biomass growth was parallel to the nitrile hydrolysis, with neither amide nor acid detectable in the culture supernatant, indicating a much more balanced catabolism and anabolism of the nitrile as a growth substrate. Only when the biomass growth was impaired in the absence of vitamins was some accumulation of an acid intermediate detectable in the ANL-iso4 culture (Table 2.2). This indicated that ANL-iso4 required the vitamins not for the nitrile degradation itself but for the assimilation of organic carbon.

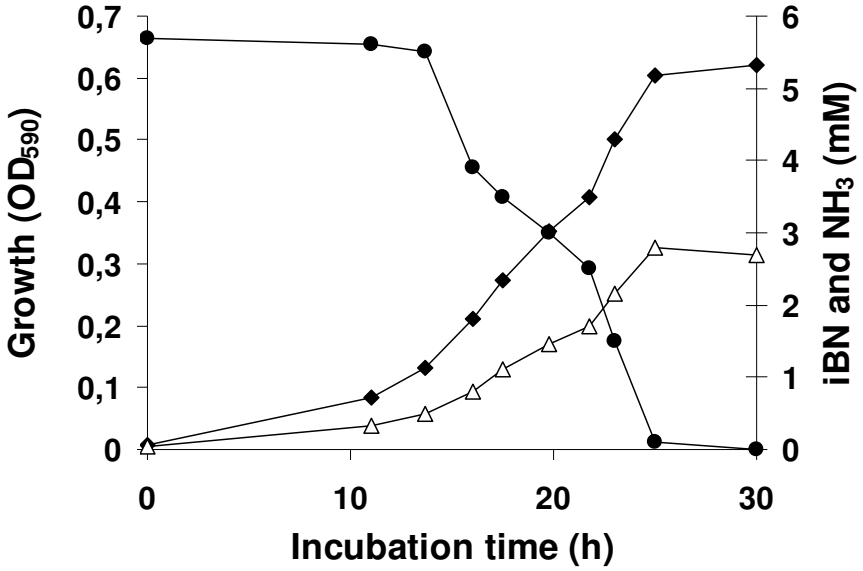


Figure 2.3 Growth and product formation with iBN as carbon, energy, and nitrogen source in batch cultures of *Bacillus* strain ANL-iso4, iBN (●), NH_3 (Δ), biomass (♦) The means of the results of two experiments with deviations of < 10 % are shown.

Influence of pH and salt on growth and iBN degradation activity

According to the results of pH and salt profiling, all four isolates belonged to moderately salt-tolerant alkaliphiles. The pH ranges for growth at 0.6 M Na^+ with iBN were 8.4 to 10.4 (optimum: pH 9.0 to 9.5) for ANL-iso2 and 7.0 to 10.25 (optimum: pH 9.0 to 9.35) for ANL-iso4. Using iBA as the substrate, the pH ranges were 8.0 to 10.5 (optimum: pH 9.5) for ANL-isoa and 7.3 to 10.3 (optimum: pH 9.0) for ANL-isoa2. The salt range for growth (M Na^+) at pH 10 was 0.1 to 2 M (optimum, 0.3 to 0.5 M) for all four isolates.

The influence of the pH on the levels of NHase and amidase activity in the two nitrile degrading strains was of particular interest. The levels of iBN and iBA hydrolysis activity were tested either with washed cells grown with iBN at pH 10 or with cell-free extract. Several significant differences between the profiles for whole cells and cell-free extract as well as between the pH profiles for the activity of ANL-iso2 and ANL-iso4 were observed (Figure 2.4). Whole cells clearly tolerated a much broader pH spectrum than the enzymes that were no longer protected by the cell membrane from the external pH condition, although in ANL-iso2 this difference was

somewhat less dramatic than in ANL-iso4. Another difference between these two organisms was the higher alkali tolerance of the nitrile-degrading enzymes in the actinobacterium.

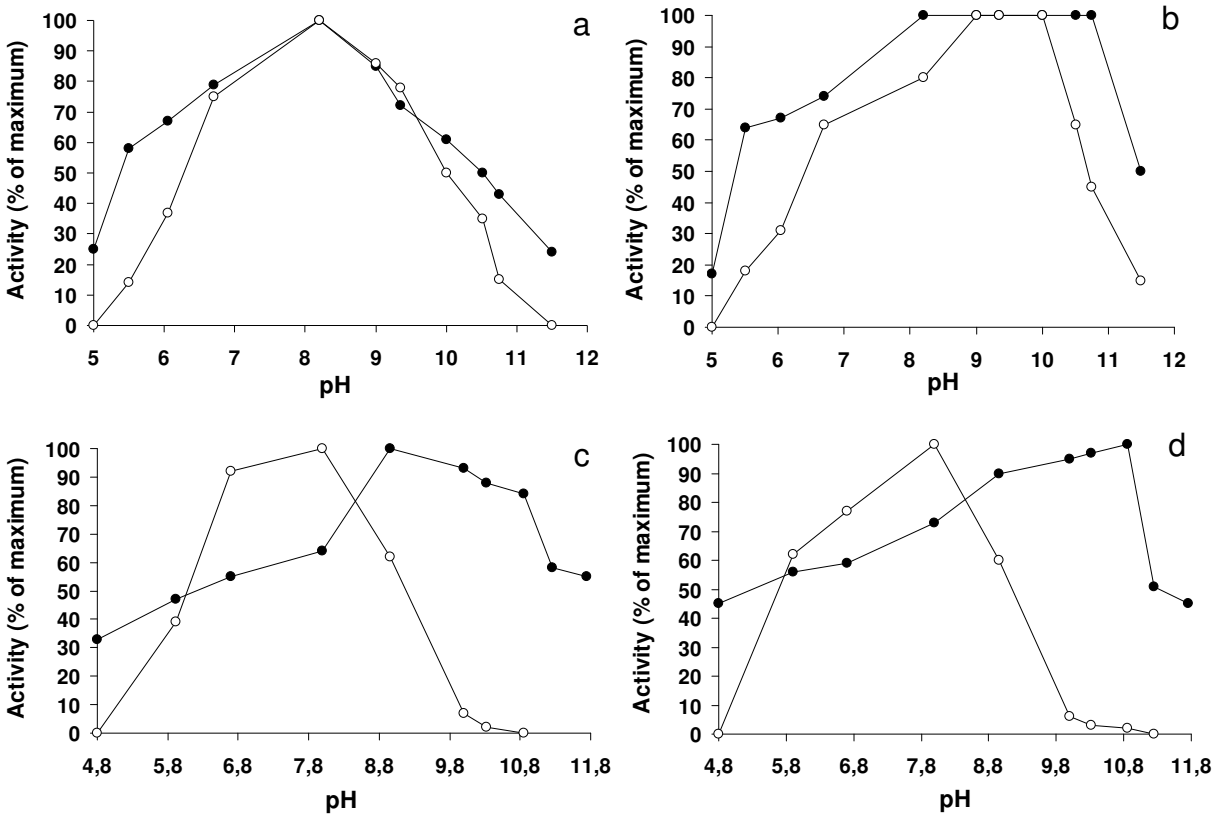


Figure 2.4 Influence of pH on the activities of the NHase/amidase systems in strains ANL-iso2 (**a, b**) and ANL-iso4 (**c, d**) in whole cell (●) and cell-free extract (○) formulation. (**a, c**) NH_3 formation from iBN. (**b, d**) NH_3 formation from iBA. The average values of the results of two independent experiments with deviations of 5 to 10 % are shown.

Metabolic versatility of the haloalkaliphilic isolates

The growth experiments revealed that the two nitrile utilising isolates, an actinobacterium and a *Bacillus* sp., can metabolise several aliphatic nitriles, most probably through NHase activity since both possessed amidase activity and both produced amides from nitriles, although in different proportions. Experiments with washed cells confirmed the presence of a NHase/amidase system in both nitrile degrading strains and also demonstrated that the actual range of substrates converted is much broader than that utilised for growth (Table 2.3).

An extremely high level of NHase activity was observed for C_4 and C_5 aliphatic nitriles, while the level of amidase activity was two to three orders of magnitude lower. In both nitrile hydrolysing strains NHase activity was induced at the same level by either iBN or iBA, while the cells grown on acetate, glucose, or peptone retained only 0 to 10 % activity.

Table 2.3 Range of substrates converted by washed cells of nitrile hydrolysing haloalkaliphiles

Substrate	Level [µmol (mg protein·min)$^{-1}$] of activity in:					
	ANL-iso2		ANL-isoa, amidase activity	ANL-iso4		ANL-isoa2, amidase activity
	NHase activity[b]	Amidase activity[c]		NHase activity[b]	Amidase activity[c]	
Acetonitrile/acetamide	6.2	< 0.1	0	4.6	< 0.1	0.1
Propionitrile/propioamide	16	0.1	1.3	33	0.1	0.1
Butyronitrile/butyramide	50	0.1	0.1	35	0.1	0.2
iBN/iBA	24	0.3	0.3	22	0.3	0.1
Valeronitrile/valeramide	58	0.2	0	31	0.3	0.3
Capronitrile/caproamide	30	0.1	0	23	0.4	0.3
Acrylonitrile/acrylamide	28	< 0.1	2.2	32	< 0.1	< 0.1
Benzonitrile/benzamide	18	< 0.1	0	3.1	<0.1	< 0.1

[a.] Cells were grown with either iBN (strains ANL-iso2 and ANL-iso4) or iBA (strains ANL-isoa and ANL-isoa2) at pH 10. Values are the averages of the results of two or three experiments with deviations within 15 %

[b.] Amide and acid formation from nitriles at pH 8

[c.] NH_3 formation from amides at pH 9 to 10

Identification of strain ANL-iso2

Strain ANL-iso2 formed flat, spreading microcolonies on the iBN alkaline agar after one month of incubation. The cells were small and non-motile rods occurring singly or in pairs under most conditions. When grown on glucose and yeast extract, chain formation was observed. The cell wall was of the Gram-positive type (Figure 2.5). Phylogenetic analysis based on 16S rRNA gene sequencing placed the nitrile utilising alkaliphile within the class *Actinobacteria* as a novel deep lineage (Figure 2.6). It formed an independent cluster together with several uncultured actinobacteria from various habitats, including soda lakes in the USA, Egypt and Russia.[20-22] However, strain ANL-iso2 showed no more than 85% 16S rRNA gene sequence

similarity to any recognised taxon. Therefore, the only safe conclusion that could be drawn from 16S rRNA gene sequence comparison was that the novel bacterium is a member of the class of *Actinobacteria*.

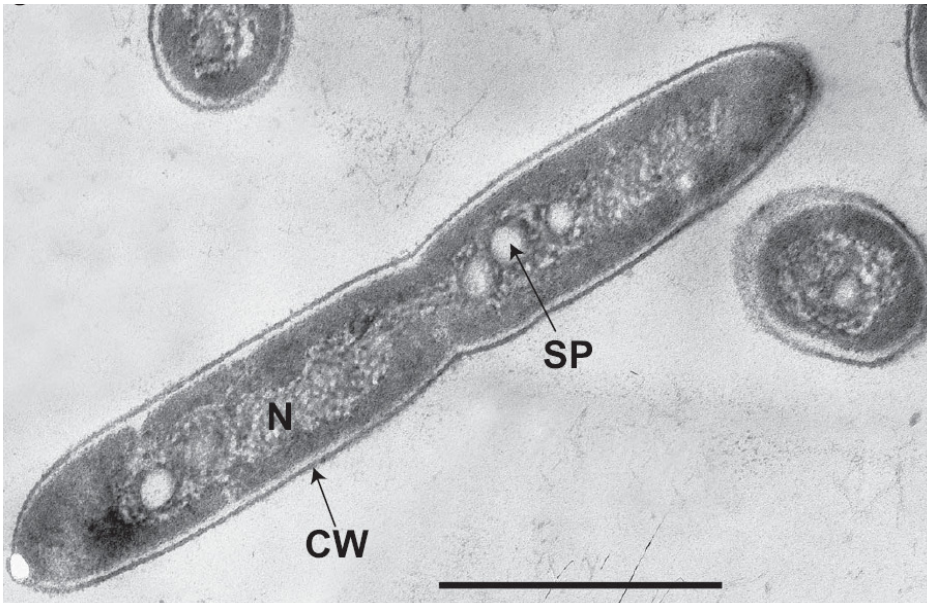


Figure 2.5 Thin section cell morphology of strain ANL-iso2; N: nucleotide, CW: cell wall, SP: inclusions of a storage polymer (most probably PHB).

At the time of writing the class of *Actinobacteria* consists of six orders, among which the order *Actinomycetales* is the most numerous and diverse (Figure 2.6). Strain ANL-iso2, despite being a typical actinobacterium in its morphotype, phylogenetically forms an independent lineage. The lack of such actinobacteria in culture might originate from their very slow growth and their specialisation on exotic toxic substrates such as nitriles. It is interesting to note that actinobacterial genera such as *Rhodococcus*, *Arthrobacter*, *Nocardia,* and *Gordonia* contain many highly active and versatile nitrile hydrolysing strains, including some that are industrially important.[4,13,23]

Strain ANL-iso2 can be regarded as the first haloalkaliphilic versatile nitrile degrader and as a representative of a novel deep phylogenetic lineage within the class of *Actinobacteria*. Based on the unique phenotypic properties and distinct phylogeny we suggest that strain ANL-iso2 represents a novel species of a new genus for which the name *Nitriliruptor alkaliphilus* gen. nov., sp. nov. is proposed and

that this forms a novel family *Nitriliruptoraceae* fam. nov. and a new order *Nitriliruptorales* ord. nov. within the class *Actinobacteria*.[♣]

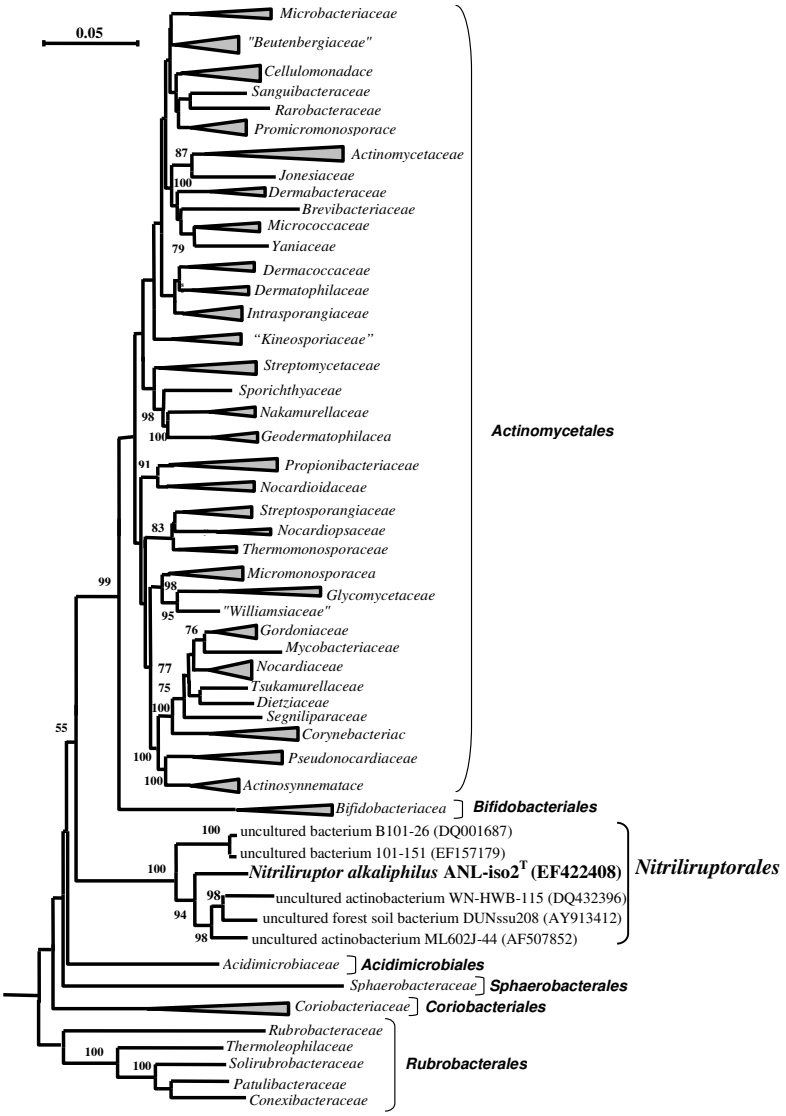


Figure 2.6 Phylogenetic position of strain ANL-iso2 and its uncultured relatives within the class of *Actinobacteria* based on 16S rRNA gene sequence analysis. The numbers at the nodes are bootstrap percentages for the clade of this group based on 1000 replications. Only values above 50% are shown. Bar: 0.05 substitutions per nucleotide position.

[♣] For a full description of the new order, family, and genus refer to: D. Y. Sorokin, S. van Pelt, T. P. Tourova, L. I. Evtushenko, *Int. J. Syst. Evol. Microbiol.* **2009**, 59, 248-253

Identification of strain ANL-iso4

Strain ANL-iso4 is a fat rod with pointed edges forming round subterminal endospores. The cells are motile using several peretrichous flagella and have a Gram-positive type of cell wall.

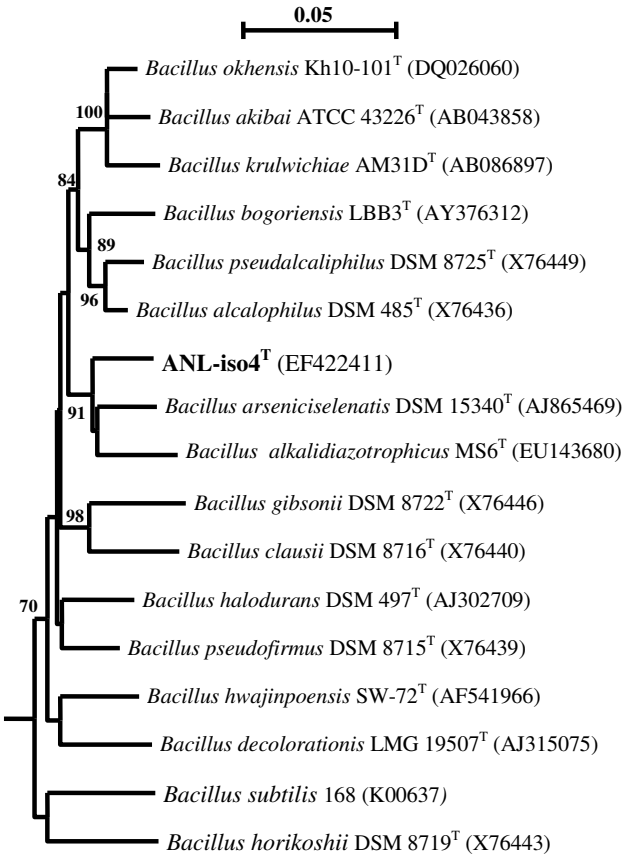


Figure 2.7 Phylogenetic tree (neighbor-joining algorithm) based on the rRNA gene sequencing showing the position of the nitrile utilising haloalkaliphile strain ANL-iso4 among the rRNA group I of the genus *Bacillus*. The numbers on the branches indicate bootstrap values above 70% after resampling 1000 times. The scale bar corresponds to sequence divergence. Bold indicates the strain used in this study.

According to the 16S rRNA gene sequence analysis the strain belongs to the rRNA group 1 of the genus *Bacillus*. It formed a separate cluster with an undescribed soil *Bacillus* sp. BA299 (98% sequence similarity) and with the haloalkaliphilic anaerobes *Bacillus arseniciselenatis* and *Bacillus alkalidiazotrophicus* as the closest validly described *Bacillus* species (95% sequence similarity) (Figure 2.7). This

clustering was stable when other tree algorithms (maximum parsimony and maximum likelihood) were applied (data not shown). Based on unusual physiological properties and phylogenetic analysis the haloalkaliphilic nitrile utilising strain ANL-iso4 is suggested as a novel species *B. alkalinitrilicus* sp. nov.[♠]

Conclusion

Our investigation of iBN degradation by soda lake sediment and soda soil microbial communities demonstrated that iBN degradation is possible under highly haloalkaline conditions and takes place through the NHase/amidase pathway (Scheme 2.1). This fact might indirectly indicate that compounds like iBN might be produced as natural substrates in natural environments, for example by the degradation of certain amino acids[2], which would be interesting to test in the future.

As is usually the case with most isolates from soda lake sediments and soda soils, the new bacteria showed typical haloalkaliphilic properties and, as such, represent a new branch of nitrile and amide hydrolysing whole cell biocatalysts. Whole cell catalysis is common practice in nitrile bioconversion, since both NHase and nitrilase are very unstable enzymes in their free forms. Therefore, further exploration of the potential applications for haloalkaliphilic nitrile-degrading bacteria and their enzymes might be interesting.

Experimental

Sediment and soil samples

A sediment mixture made of 10 samples from Kulunda Steppe (south-western Siberia, Altai, Russia) soda lakes and a mixture of 20 samples of solonchak soda soils (Kulunda Steppe and north-eastern Mongolia) were used as the inocula to be

[♠] For a full description of the new species refer to: D.Y. Sorokin, S. van Pelt, T.P. Tourova, *FEMS Microbiol. Lett.* **2008**, 288, 235-240.

enriched for culturing iBN degrading haloalkaliphiles. The pHs, alkalinities, and total salinities of the lakes were within the ranges of 9.5 to 11.05, 0.2 to 4.0 M, and 50 to 400 g L^{-1}, respectively (20). The pHs of water extracts from the solonchak soil samples varied from 9.5 to 10.8, the total alkalinity from 0.05 to 1.2 M, and the total salt content from 30 to 200 g kg^{-1}. The dominant ions in both habitats were Na^{+}, Mg^{2+}, HCO_3^{-}/CO_3^{2-}, Cl^{-1}, and SO_4^{2-}.

Media compositions and growth conditions

A mineral medium based on sodium carbonate buffer at pH 10 and 0.6 M total Na^{+}, containing 22 g L^{-1} Na_2CO_3, 8 g L^{-1} $NaHCO_3$, 6 g L^{-1} NaCl, 0.5 g L^{-1} K_2HPO_4, was used for the enrichment and pure culture experiments. The pH of this medium was stable even after prolonged incubation. After being sterilised, the medium was supplemented with 1 mL L^{-1} trace metal solution,[24] 1 mM $MgSO_4$, and 0.1 mg L^{-1} of filter-sterilised vitamin B_{12}.

The enrichment was performed in 100 mL serum bottles closed with rubber septa (to prevent substrate loss) containing 20 mL medium and 1 mL sediment or 1 g soil. In the case of nitriles and their corresponding amides, these compounds were used as both C- and N-sources, while in the case of carboxylic acids, the medium was supplemented with 4 mM NH_4Cl. iBN to a final concentration of 2 to 10 mM was added directly to each culture vessel before the vessel was closed. In the case of the solid medium (the solidifying agent was Noble agar; Difco), iBN was added after the medium was cooled down to 50 °C, to prevent excessive loss of substrate. Liquid cultures were incubated on a rotary shaker at 100 rpm and 28 °C and were periodically checked for ammonia production. When the ammonia concentration reached 2 mM, the culture was transferred into a new medium at a 1:100 dilution. After 3 to 4 successful 1:100 transfers, the culture was serially diluted up to 10^{-11}. The culture from a maximal positive dilution was plated onto solid medium, either by surface spreading or by the agar-shake technique. The plates were incubated in closed jars for 30 days. Separate colonies were placed into 5 mL liquid medium with iBN or isobutyroamide (iBA) in 30 mL serum bottles closed with rubber septa. Positive cultures were plated again to check for purity.

Growth experiments with pure cultures were performed in 250 mL closed serum bottles with 50 mL liquid on a rotary shaker at 100 to 150 rpm and 30 °C. Substrates were used at a 5 to 20 mM concentration. The growth was monitored by assessing the optical density, and the degradation of nitriles was followed by assessing the level of ammonium production, the disappearance of the substrate, and the formation of intermediates. The pH profiling of the growth of the cultures was done in medium containing 0.6 M total Na^+ either as NaCl (pH 6.5 to 8.0) or $NaHCO_3/Na_2CO_3$ (pH 8.5 to 11.0).[25] The salt dependence for the growth of the cultures was investigated in a range of sodium carbonate-based media containing 0.1 to 3.0 M total Na^+ at pH 10.

Experiments with washed cells and cell-free extracts

To determine the substrate profiles and activities of the pure cultures and the influence of pH and salt concentration on the activities, cells grown at pH 10 and 0.6 M Na^+ with various substrates were harvested, washed, and resuspended in 0.5 M sodium carbonate buffer, pH 9, at a cell density of 20 to 25 mg mL^{-1} protein. To obtain a cell-free extract, the same cell suspension was sonicated, followed by the removal of unbroken cells by centrifugation.

Two types of activity tests were performed. To determine the level of one of the final hydrolysis products, ammonia, the washed cells and cell-free extract were incubated in 2 mL Eppendorf tubes with 1 mL of reaction mixture at 30 °C. The tubes were shaken horizontally at 200 rpm. The formation of ammonium was checked regularly in 50 µL samples after rapid centrifugation. Activity measurements to determine the levels of nitrile consumption and amide and acid production were performed in 1.5 mL Eppendorf tubes on a ThermoTWISTER comfort (QUANTIFOIL Instruments). To 1.5 mL buffer (0.5 M $NaHCO_3$/0.1 M NaCl, pH 8 to 8.5) 0.5 to 20 µL of pure nitrile was added directly from the source liquid and the reaction mixture was shaken horizontally (700 rpm) at 21 °C. The reaction was initiated by the addition of 5, 10, or 20 µL of cell suspension with a known protein concentration (20 to 25 mg mL^{-1} protein). Regular samples were taken and quenched in 1 M HCl solution. After centrifugation of the denatured protein, the supernatant was either injected directly

into a high performance liquid chromatography column or diluted first with MilliQ water when necessary.

Analytical procedures

The protein concentration was measured by the Lowry method. The ammonium concentration was determined by the phenol-hypochlorite method according to Weatherburn.[26]

Nitriles, amides, and carboxylic acids were detected by high performance liquid chromatography. All compounds were detected by using a Merck Chromolith SpeedROD RP-18e column (50 X 4.6 mm), except for acetonitrile, acetamide, and acetic acid. Acrylonitrile, methacrylonitrile, butyronitrile, iBN, valeronitrile, nicotinonitrile, and their corresponding amides and acids were separated by using an eluent of MilliQ water (98.9 %), acetonitrile (1 %), and acetic acid (0.1 %). Benzonitrile, phenylacetonitrile, capronitrile, and their corresponding amides and acids were separated by using an eluent of MilliQ water (89.9 %), acetonitrile (10 %), and acetic acid (0.1 %). Propionitrile and its corresponding amide and acid were separated by using an eluent of MilliQ (99.9 %) and acetic acid (0.1 %). All separations on the SpeedROD were carried out at 21 °C and with a flow rate of 1 mL min^{-1}. Acetonitrile, acetamide, and acetic acid were detected by using a Phenomenex Rezex ROA-organic acid H^+ column (300 X 7.80 mm; 8 microns) with an eluent consisting of MilliQ water with 0.01 M trifluoroacetic acid (0.6 mL min^{-1}) and a column temperature of 60 °C. The aliphatic nitriles were detected by using a Shimadzu RID 10A refractive index detector. All other compounds were detected by using a Shimadzu SPD-10A VP UV-VIS detector. A wavelength of 210 nm was used to detect all aliphatic amides and acids, and a wavelength of 230 nm was used to detect all aromatic amides and acids.

Phase-contrast microphotographs were obtained by using a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For electron microscopy, cells were fixed with glutaraldehyde (final concentration 3 %, vol/vol) and stained with 1 % (wt / vol) uranyl acetate for positive contrast. For thin sectioning, the cells were fixed in 1 % (wt / vol) OsO_4 and 0.5 M NaCl for 3 h at room temperature, washed, stained

overnight with 1 % (wt / vol) uranyl acetate, dehydrated in an ethanol series, and embedded in Epon resin. The thin sections were stained with 1 % (wt / vol) lead citrate. The isolation of the DNA and subsequent determination of the G + C content and the DNA-DNA hybridisation were performed by using the thermal denaturation / reassociation technique.[27,28] Genomic DNA for phylogenetic analysis was extracted from the cells by using an UltraClean soil DNA extraction kit (MolBio Laboratories, United States) following the manufacturer's instructions. The 16S rRNA genes were amplified using general bacterial primers. The PCR products were purified from low melting agarose by using a Wizard PCR Prep kit (Promega, United States) according to the manufacturer's instructions. Sequencing was performed by using a BigDye Terminator v.3.1 sequencing reaction kit on an ABI 3730 DNA automatic sequencer (Applied Biosystems, Inc., United States). The sequences were first compared with those stored in GenBank by using the BLAST algorithm. The sequences were aligned with those from GenBank by using ClustalW. Phylogenetic trees were constructed with four different algorithms using the TREECONW software package.[29] Tree topology and evolutionary distances were calculated by the neighbour-joining method with the corrections of Jukes & Cantor (1969).

Nucleotide sequence accession numbers

The GenBank accession numbers for the 16S rRNA gene sequences of the strains ANL-iso2, ANL-iso4, ANL-isoa, and ANL-isoa2 are EF422408, EF422411, EF422409, and EF422410, respectively.

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3

Nitriliruptor alkaliphilus: Characterisation of a Nitrile Hydrating Biocatalyst

Abstract

In this chapter, the induction of nitrile hydratase activity in *Nitriliruptor alkaliphilus* was examined in more detail. Without the presence of a nitrile, carboxylic amide, or carboxylic acid in the fermentation broth, very little or no nitrile hydratase activity was detected. It was found that the inducing agent concentration during growth should be maintained at a high level. A maximum nitrile hydratase activity of 30 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ for the hydration of hexanenitrile was achieved when *N. alkaliphilus* cells were cultivated on isobutyronitrile or isobutyramide, added in four separate portions of 2.5 mM, as the only C- and N-source. The whole cell nitrile hydrating biocatalyst is most active on C_4 and C_5 straight chain saturated aliphatic nitriles, but also easily accepts saturated and unsaturated branched aliphatic nitriles, as well as (hetero)aromatic and arylaliphatic nitriles, except for phenylacetonitrile. Although highly stable at low temperatures, the cells rapidly start losing activity at temperatures higher than 37 ^oC. The pH optimum of the free enzyme is 8, while the whole cells have a broad pH optimum from 7 - 10. An ammonium sulfate fractionation combined with an anion exchange purification purified the cell-free extract by a factor of 7.3 and removed all amidase and esterase activity. The α- and β-subunits have a molecular weight of around 26 and 28 kDa and the characteristic C**T**LCSC domain for Co-type nitrile hydratases was identified by a PCR based screening method using degenerate primers.

Introduction

The enzymatic conversion of a nitrile into the corresponding acid is one of the pathways of nitrile metabolism in Nature. This reaction can be catalysed either by a nitrilase or by a nitrile hydratase/amidase coupled system.[1] Nitrile hydratase (NHase, E.C. 4.2.1.84) catalyses the conversion of a nitrile into the corresponding carboxylic amide and was first discovered 30 years ago in studies on the microbial degradation of toxic cyano-group-containing compounds.[2] During the last two decades, NHases have found industrial applications in the production of acrylamide and nicotinamide on a multi-ton scale.[3]

NHases are enzymes that are mainly present in bacteria.[1] They are described to occur in species belonging to the phyla *Proteobacteria*, *Actinobacteria*, *Cyanobacteria* and *Firmicutes* in habitats ranging from soil,[4] via coastal marine sediments[5] and deep sea sediments,[6] to geothermal environments.[7] NHases can be divided into two different groups: ferric NHases and cobalt NHases. Ferric NHases contain a non-heme Fe (III) ion in the active site,[8] while cobalt NHases contain a non-corrinoid Co (III)[9] ion in the active site. All NHases consist of α- and β-subunits in equimolar amounts, each of a size between 23-29 kDa. The different NHases isolated so far have wide-ranging physicochemical properties and substrate specificities.[1] Most results showed that the Fe-type NHases preferentially hydrate small aliphatic nitriles, whereas the Co-type NHases exhibit a relatively high affinity for aromatic nitriles.[10]

In the preceding Chapter the discovery of the new nitrile hydrolysing bacterium *Nitriliruptor alkaliphilus,* by enrichment on isobutyronitrile from soda lakes, was described. In this Chapter the induction of NHase activity in this organism is studied in more detail. The properties of *N. alkaliphilus* as a whole cell nitrile hydrating biocatalyst are described, as well as the purification and preliminary biochemical characterisation of this NHase enzyme.

Results and Discussion

The induction of NHase activity

The regulation of NHase activity in *N. alkaliphilus* was investigated in an attempt to increase the activity of this biocatalyst. NHase expression can be both constitutive as well as inducible.[1] The induction of the NHase/amidase enzymatic pathway of nitrile hydrolysis takes place during cultivation and is typically performed by the NHase or amidase substrates, products, or their structural analogues. Different inducing agents, like urea[11] and caprolactam,[12] are also described in literature. In order to investigate the inducibility of the NHase/amidase enzymatic pathway in *N. alkaliphilus*, the cells were grown on different growth substrates, after which the relative activities of these enzymes were determined by NH_3 formation (Table 3.1).

Table 3.1 The expression of the NHase/amidase enzymatic pathway in *N. alkaliphilus* for different growth substrates was assayed by the conversion of iBN and IBA.

Growth substrate	Relative NH_3 formation (%)	
	From iBN as the substrate	From iBA as the substrate
iBN	100	100
iBA	90	90
Propionitrile	90	90
Acetate	0	0
Yeast extract	10	10

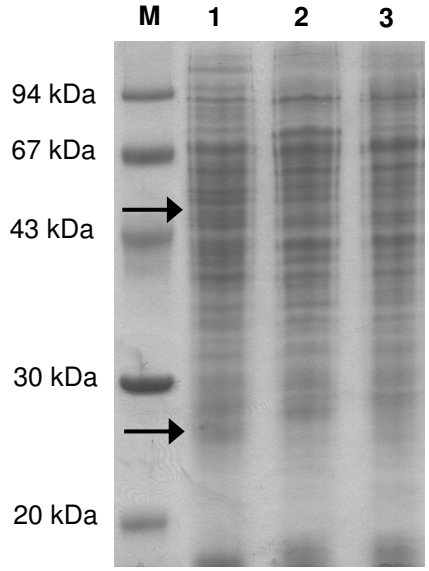


Figure 3.1 Expression of a NHase and amidase in *N. alkaliphilus* (12 % SDS-PAGE of the total protein content). (**1**) Cells grown with iBN (activity = 100 %), (**2**) cells grown with acetate (activity = 0 %), (**3**) cells grown with yeast extract (activity = 10 %).

The NHase/amidase enzymatic pathway was induced by isobutyronitrile (iBN), isobutyramide (iBA), as well as by propionitrile. Without the addition of a nitrile or amide compound no activity was found. On the SDS-PAGE gel of the total protein content, a vague extra band around 27-28 kDa was found for cells induced with iBN,

which is the expected molecular weight for the α- and β-subunits of a NHase.[1] A small extra band was also detected around 50 kDa, which indicates the presence of an amidase (Figure 3.1).[1]

During the cultivation of *N. alkaliphilus* on iBN as the only C- and N-source with a 10 mM starting concentration, the specific activity of the cells decreased during the exponential growth phase (Figure 3.2). Nevertheless, the highest total activity was reached during the beginning of the stationary growth phase.

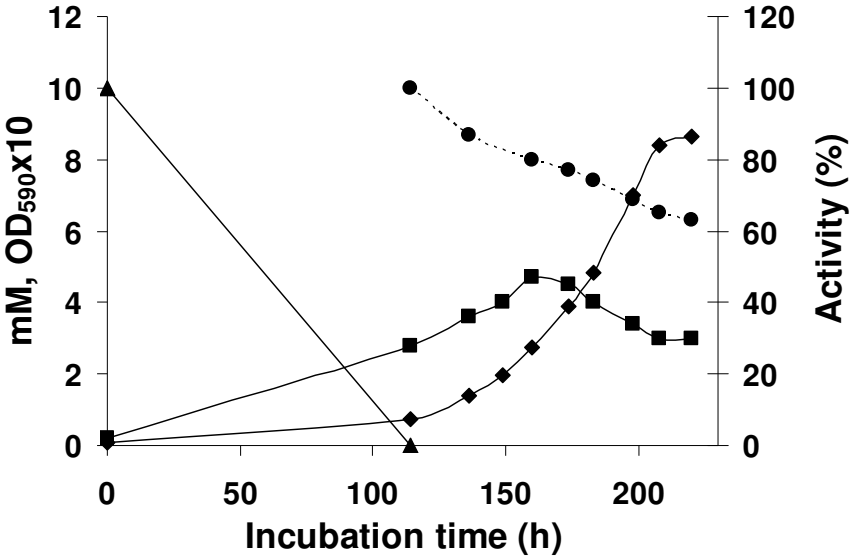


Figure 3.2 Dependence of the relative nitrile hydration activity on the growth phase and isobutyronitrile (iBN) concentration. The activity is based on the NH_3 production from iBN. Biomass (♦, OD_{590}x10), iBN (▲, mM) ,ammonia (■, mM), relative activity (●, %).

This decrease in specific activity may be related to a decrease in the concentration of the inducing agents iBN and iBA (see also Figure 2.2, Chapter 2), indicating that the concentration of these inducing agents should be kept high during growth. This hypothesis was confirmed when the induced final total NHase activity was ~ 30 % higher when iBN or iBA was added in 4 separate portions of 2.5 mM to the fermentation broth instead of in one portion of 10 mM. The activity completely disappeared when the culture was grown for one night without the presence of inducing agents, which indicates that the NHase protein was broken down by the cells when it was no longer functional.

Because of the slow growth characteristics of *N. alkaliphilus* (Figure 3.2) an attempt was made to grow the cells first on a rich medium containing peptone, after which the NHase activity was induced by a 5 hour incubation period of the cells with different inducing agents (Table 3.2). Although the late stage induction of the *N. alkaliphilus* cells with iBN and iBA resulted in a higher NHase activity compared to when isobutyrate (iB) or no inducing agent was used, the activity was still substantially lower compared to cells cultivated on iBN or iBA as the only C- and N-source in 4 portions of 2.5 mM. *N. alkaliphilus* cells that were grown first on iB as a non-volatile and non-toxic growth substrate had a higher NHase activity, which could be slightly raised by the late stage induction with iBN or iBA. However, iB was less effective as an inducing agent during growth than iBN or iBA. Since the relative acivities were lower and the specific growth rate did not significantly improve, the cultivation method using late stage induction was abandoned.

Table 3.2 Effect of a 5 hour incubation of *N. alkaliphilus* cells with different inducing agents on the NHase activity. The cells were first grown on either a rich medium with peptone or on iB.

Inducing agent	Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)[a]	
	Grown on a rich medium with peptone	Grown on 10 mM iB +NH_4Cl
None	0.13	9.16
2 mM iB	0.17	-
3 mM iBA	4.19	11.97
3 mM iBA + Co^{2+} [b]	5.51	11.86
3 mM iBN	3.06	11.43
3 mM iBN + Co^{2+} [b]	4.45	11.59

[a] The activity was determined by the hydration of hexanenitrile at pH 8 and 21 °C. The NHase activity is between 27 and 30 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ when the cells are grown on iBN or iBA as the only C- and N-source in 4 portions of 2.5 mM.

[b] A 5 x higher concentration of $CoCl_2 \cdot 6H_2O$ than normally used during growth (1.25 $mg \cdot L^{-1}$ = ~ 5.3 µM).

An increased Co^{2+} concentration next to the addition of iBN or iBA in the late stage induction experiments seemed to have a positive effect on the NHase activity when the cells were first grown on a rich medium containing peptone. However, when the cells were first grown on iB this positive effect is no longer visible. Different

Co^{2+} concentrations (1.1, 2.1, 3.2, 4.2, and 5.3 µM $CoCl_2 \cdot 6H_2O$) during the growth on 4 portions of 2.5 mM iBA similarly had no positive effect on the activity, indicating that the cobalt concentration was not a limiting factor for NHase activity under these growth conditions. $CoCl_2 \cdot 6H_2O$ concentrations higher than 1.25 $mg \cdot L^{-1}$ proved toxic for the *N. alkaliphilus* cells and inhibited growth. In contrast, $CoCl_2 \cdot 6H_2O$ concentrations as high as 10 $mg \cdot L^{-1}$ have been used in the growth of the extremely active *R. rhodochrous* J1 and *P. thermophila*.[13,14] Since the iron concentration in the medium was already high (~ 7.2 µM), no growth experiments were carried out with an increased iron concentration.

Although iBN and iBA were equally effective as inducing agents, the growth of *N. alkaliphilus* on iBN is inconvenient since this growth substrate is very toxic and highly volatile. Therefore iBA was the inducing agent of choice for further fermentations and was added portionwise to the medium in aliquots of 4 times 2.5 mM. Using this growth recipe, a NHase activity in the cells of around 30 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ was achieved for the hydration of hexanenitrile.

Properties of the whole cell biocatalyst

A concentrated cell suspension of *N. alkaliphilus* could be stored at 4 ^oC in a pH 9 carbonate buffer for months without a significant activity loss. The activity loss after 4 months was only 10% and there was no sign of cell lysis. In contrast, concentrated cell suspensions of *Rhodococcus* sp. R312 and *Bacillus alkalinitrilicus* completely lost their NHase activity in 2-3 weeks and 3-4 days respectively because of cell lysis and subsequent deactivation of the enzyme. Although the *N. alkaliphilus* NHase activity was maintained at low temperatures, the activity was rapidly lost during incubation at temperatures higher than 37 ^oC (Figure 3.3A) and an immediate and complete loss of the NHase activity was found at temperatures of 60 ^oC and higher.

Although the pH optimum of the cell free NHase is 8, the whole cell NHase activity deviates only marginally from this optimum reaction rate in the range of pH 7 - pH 10 (Figure 3.3B). The ability of the *N. alkaliphilus* cells to maintain a pH gradient across the cell membrane facilitates this broad pH optimum. The stability of the whole cell NHase activity at elevated pH values is high. During a one hour incubation

of *N. alkaliphilus* cells at pH 11, activity loss through deactivation was 13 %, while no noticeable activity loss was detected at the pH values of 9 and 10.

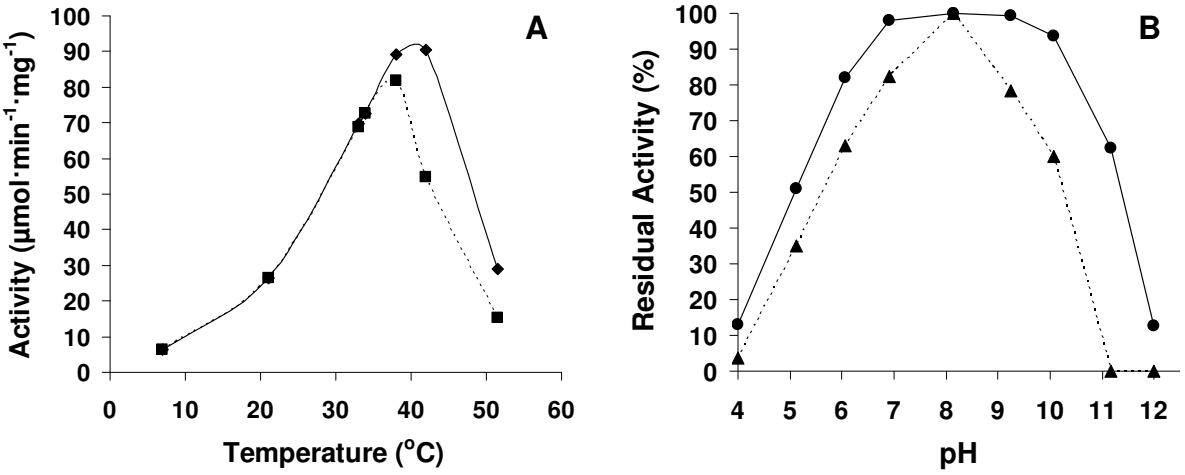


Figure 3.3 (**A**) Temperature profile determined by the whole cell hydration of hexanenitrile after a 0 min. (♦) and 5 min. (■) incubation at different temperatures in a buffer of pH 8 (0.01 M Tris-HCl). (**B**) pH profile determined by the whole cell (●) and cell free extract (▲) hydration of hexanenitrile after a 20 minute incubation at different pH and 21 °C (pH 4-6: 0.01 M citrate buffer, pH 7-9: 0.01 M Tris-HCl buffer, pH 10-12: 0.01 M phosphate buffer).

The NHase from *N. alkaliphilus* was the most active on C_4 and C_5 straight chain, saturated, aliphatic nitriles (Table 3.3). Branched aliphatic nitriles or aliphatic nitriles with a chain length lower than C_4 or higher than C_5 were hydrated with a lower relative activity. Aromatic nitriles and arylaliphatic nitriles, with the exception of phenylacetonitrile, were also well-accepted by the enzyme. The amidase activity in the cells is 2-3 orders of magnitude lower than the NHase activity, which is convenient from a downstream processing point of view when the desired product is the amide.

Table 3.3 Activities of the *N. alkaliphilus* NHase for the hydration of several structurally different nitriles.

Substrate	Activity[a]	Substrate	Activity[a]
Acetonitrile	6[b]	Methacrylonitrile	23
Propionitrile	16	Benzonitrile	18
Butyronitrile	50	3-Cyanopyridine	9
Isobutyronitrile	24	Phenylacetonitrile	0.84 – 0.47[c]
Pentanenitrile	58	2-Phenylpropionitrile	8
Hexanenitrile	30	2-Azidopentanenitrile	15-16
Adiponitrile	29[e]	2-Azidophenylpropionitrile	7
Acrylonitrile	28[b]	α,α-disubstituted cyanohydrin acetates	- [d]

[a.] Activity (μmol·min^{-1}·mg protein^{-1}) was determined at pH 8 (0.01 M Tris-HCl) and 21 °C with nitrile concentrations between 3 – 100 mM. Amidase activity in the whole cells was 2-3 orders of magnitude lower than the NHase activity.

[b.] The apparent K_m = 50 mM

[c.] Depending on the starting nitrile concentration (4.8 – 13.2 mM). Activity was inhibited by higher nitrile concentrations.

[d.] The main product formed is the corresponding ketone.

[e.] Activity of the hydration to 5-cyanopentaneamide.

Since acrylamide is an important industrial compound, the production of this compound using *N. alkaliphilus* whole cells was further investigated. Although the initial activities for the hydration of acrylonitrile were constant in the region between 200 and 600 mM of starting nitrile concentration, high concentrations of acrylonitrile have a detrimental effect on the stability of the whole cell biocatalyst at 21 °C (Figure 3.4A). Although the whole cell NHase activity is lower at 10 °C, biocatalyst stability drastically improves (Figure 3.4B). The deactivation of the NHase may be caused by a reaction of the highly reactive acrylonitrile with essential amino acid residues of the enzyme. Carrying out the reaction at a lower temperature probably slows this reaction down.

Another industrially relevant substrate is adiponitrile. The primary hydration activity of the NHase for adiponitrile, forming 5-cyanopentanamide, was similar to that for hexanenitrile. After the consumption of all adiponitrile (> 98% conversion), only approximately 10 % of the formed product consisted of the undesired double hydrated product adipamide. Although the NHase from *N. alkaliphilus* showed a

reasonable regioselectivity for adiponitrile (90 %), it cannot compete with the regioselectivity of the NHase from *Pseudomonas chloraphis* B23 (96 % selectivity at 97 % conversion, Chapter 1). Although of lower value, the double hydrated product adipamide is also a useful chemical compound. During a fed-batch reaction of *N. alkaliphilus* cells with adiponitrile at 5 °C, adipamide started precipitating from the reaction mixture, thus preventing further hydrolysis to the carboxylic acid. Crystals of adipamide have a higher density than water and formed a neat layer of crystals on the bottom of the reaction vessel. Pure adipamide (1 gram, > 98%) could easily be produced using this method with high yield (98-99 %), without optimisation of the reaction conditions using 6 mg of protein in the form of whole cells in 6 mL of buffer. The product was easily isolated using a P4 glass filter.

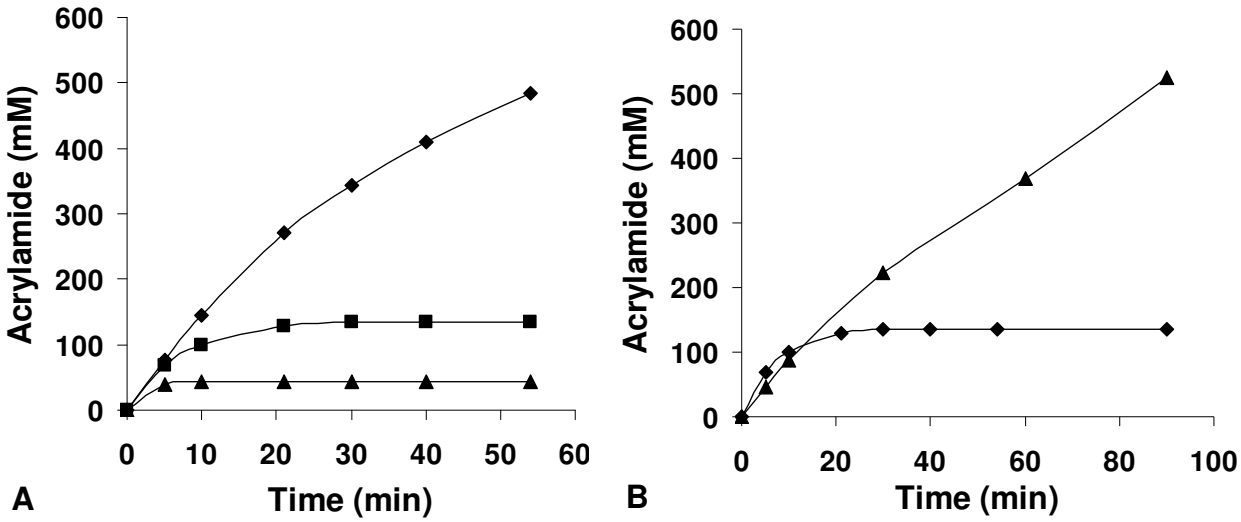
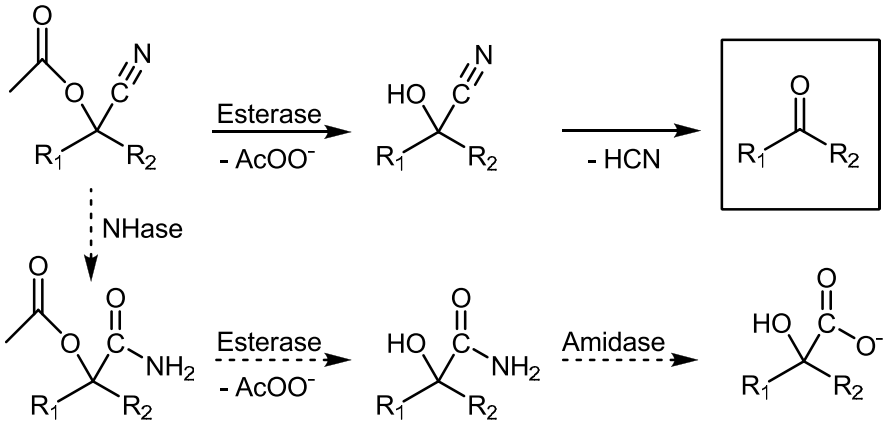


Figure 3.4 (**A**) Acrylamide production using a starting acrylonitrile concentration of 485 (♦), 921 (■), and 1340 mM (▲) at 21 °C in a pH 8 carbonate buffer with a protein concentration of 450 mg·L^{-1}. (**B**) Acrylamide production using an acrylonitrile starting concentration of 921 mM at 10 °C (▲) and 21 °C (♦) in a pH 8 carbonate buffer with a protein concentration of 450 mg·L^{-1}.

The whole cell hydration of several aromatic and aliphatic α,α-disubstituted cyanohydrin acetates resulted in only a small amount of the stable amide products. The sterically hindered nitrile was only reluctantly accepted by the NHase, while the esterase activity present in the cells of *N. alkaliphilus* cleaved the acetate group with a higher relative activity, resulting in the formation of a tertiary unprotected

cyanohydrin. These tertiary cyanohydrins were highly unstable in water and immediately decomposed in the corresponding ketone and HCN (Scheme 3.1).



Scheme 3.1 In an attempt to hydrate α,α-disubstituted cyanohydrin acetates, the main product formed was the corresponding ketone, because of the presence of an uncharacterised esterase activity in the whole cells of *N. alkaliphilus* that had a higher relative activity compared to the NHase for these substrates.

Cell-free extract storage stability

Preceding the enzyme purification, the storage stability of cell-free NHase was investigated. It is known that NHases are very unstable enzymes in cell-free formulation, although no clear reason for this instability can be found in the literature.[15,16] The cells of *N. alkaliphilus* are tough and hard to lyse. Sonication of the cells as well as incubation of the cells in pure water was not effective, which is unusual for haloalkaliphilic bacteria. Deactivation of the NHase caused by the disruption of the cells in a cell disruptor at 2 Kbar was approximately 8.5 % per volume♣. After disruption of the cells the stability of the NHase in cell-free extract was studied under different conditions.

Although using a 10 x diluted Tris-HCl buffer improved the storage stability of the cell-free NHase significantly, enzyme storage stability was still poor without any further additions (Figure 3.5). Contrary to reports in the literature,[16-18] the addition of

♣ Volumetric activity of cell suspension vs. volumetric activity of extract after disruption.

butyric acid (20 and 40 mM) to the cell-free extract did not have any noticable effect on the stability of this NHase. Storing the NHase cell-free extract under argon without the presence of oxygen or in the presence of different concentrations of various metal ions also did not improve the enzyme storage stability. The ability of glycerol to stabilise enzymes is a widely recognised phenomenon.[19] Glycerol did not only stabilise the NHase from *N. alkaliphilus* during storage at -80 °C but also significantly enhanced the stability of the enzyme during storage at 4 °C (Figure 3.5). Another method for the efficient preservation of NHase activity during storage at 4 °C is by the precipitation of the enzyme in the cell-free extract with a saturated ammonium sulfate solution of pH 8 or by the preparation of NHase cross-linked enzyme aggregates (CLEAs, Chapter 5).

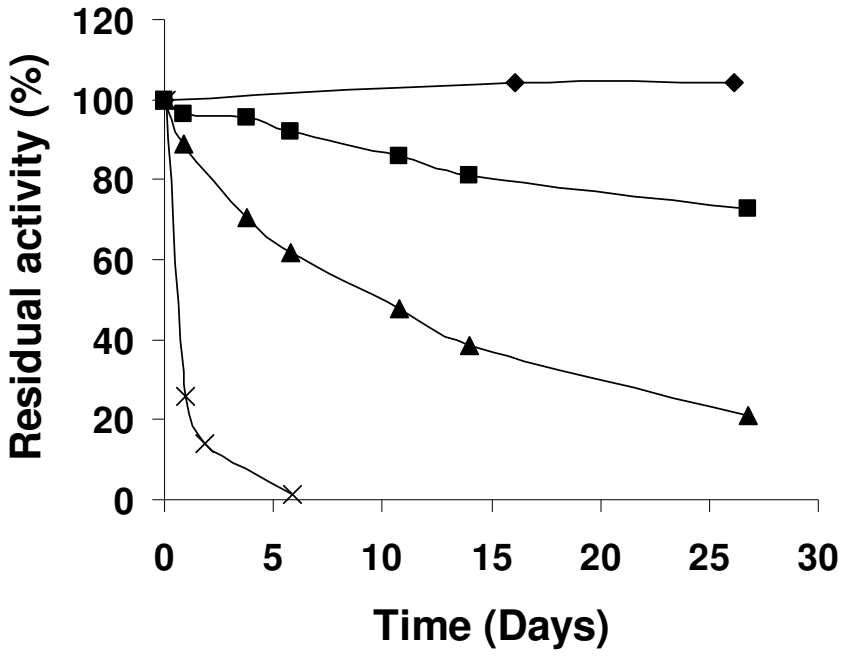


Figure 3.5 Cell-free extract stability during storage at 4 °C in: 0.1 M Tris-HCL pH8 (x), 0.01 M Tris-HCl pH 8 (▲), 0.01 M Tris-HCl pH 8 + 10 % glycerol (■), 0.01 M Tris-HCl pH 8 + 20 % glycerol (♦).

Cell-free extract purification

The undesirable esterase and amidase activities in the cell-free extract of *N. alkaliphilus* were removed by partial purification (Table 3.4, Figure 3.6).

Table 3.4: Purification and stabilisation of the cell-free NHase from *N. alkaliphilus*

Step	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)[a]	Activity yield (%)[b]	Purification (-fold)[c]
Crude extract	140	8397	60	100	1
$(NH_4)_2SO_4$ [d]	57	5063	89	60	1.5
DEAE[e]	8.1	3553	437	42	7.3
Storage[f]	7.6	2715	358	32	

a. One unit is equal to the hydration of 1 µmol·min^{-1} of hexanenitrile.

b. Activity yield indicates the relative value of the total activity of crude extract.

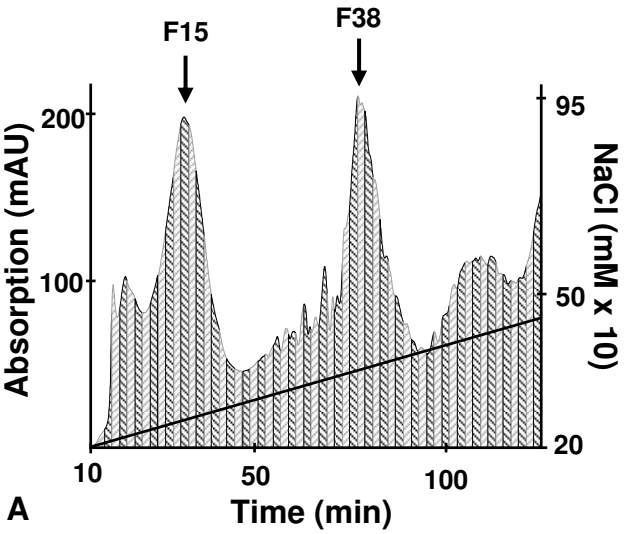
c. Purification indicates the relative value of the total specific activity of cell-free extract.

d. An ammonium sulfate cut between 41 and 67 %.

e. DEAE Sepharose Fast Flow

f. Stabilisation for storage in a 75% saturated ammonium sulfate solution of pH 8 + 30 mM butyric acid.

A 7.3-fold purification was achieved by an ammonium sulfate cut combined with an anion exchange purification using a DEAE sepharose column. It was possible to separate the amidase and NHase enzymes completely on the DEAE sepharose column (Figure 3.6). The shapes of the peaks in the protein elution profile (Figure 3.6A) match nicely with the distribution of NHase activity (Figure 3.6B) and the bands on the SDS-Page gel (Figure 3.6C). After the pooling of fractions 36 - 41 and subsequent concentration of these fractions, activity tests on hexaneamide proved that amidase activity was completely absent. Reactions of the purified NHase with α,α-disubstituted cyanohydrin acetates demonstrated the absence of esterase activity. The molecular weight of the amidase is around 50 kDa. The molecular weight of the NHase α- and β-subunit are around 26 and 28 kDa respectively. The partially purified NHase from *N. alkaliphilus* is probed for its enantioselectivity in Chapter 4.



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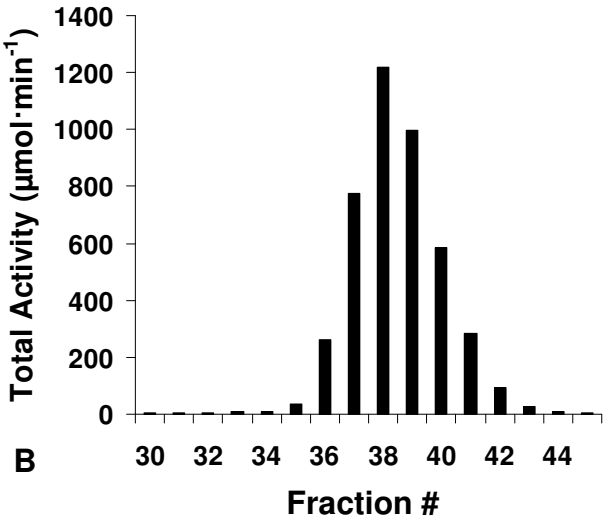

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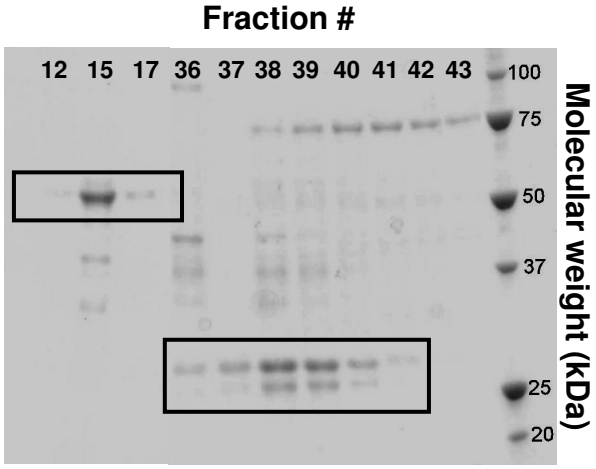

C

Figure 3.6 (**A**) Protein elution profile at 280 nm. The position of fraction 15 and fraction 38 is indicated in the graph. (**B**) Total NHase activity for the fractions 30-45. Amidase activity was only detected in fraction 12-17 with a maximum in fraction 15. (**C**) 4-12 % SDS-PAGE gel of the fractions containing amidase and NHase activity.

Sequence determination of the NHase gene

In order to determine the sequence of the NHase gene, degenerate primers were employed to find NHase-like genes in the genome of *N. alkaliphilus*. Different combinations of forward and backward primers were used in a PCR method as was described before by several groups.[20,21] Only primer NHBI B together with NHBI C (Table 3.5, experimental part) gave a band of approximately 150 bps, which is the expected size for this combination. Sequencing of this band revealed a fragment of 155 bps. The sequence of this fragment was used to develop PCR and sequencing primers for a gene walking protocol.[22] The PCR with the forward primer resulted in a large band that could be sequenced with two primers. Unfortunately, the PCR with the reverse primer did not result in large fragments, only 400 bps could be determined in front of the degenerate PCR part (Figure 3.7, experimental part). Different PCR protocols, or new PCR and sequencing primers, did not result in a further sequence determination. All sequences dropped after the same point in the sequence. Upon the investigation for possible genes in the obtained sequence it was obvious that the sequence of the α-subunit is probably 75 % complete, since the sequence is very similar to other α-subunits. Furthermore, the current size of the Open Reading Frame of the proposed protein (19.3 kDa) is close to the apparent size on the SDS-gel (26-28 kDa). The sequence of the α-subunit contains the typical Co-type NHase signature C**T**LCSC. Behind the α-subunit there are three open reading frames present of around 12 kDa, but none of these resemble any known β-subunit or activator protein. In addition, BLAST alignment of these proteins did not result in any hits to a known protein. This suggests that the activator protein and β-subunit are located in front of the α-subunit, which is seen in some other NHase operons as well. As we did not succeed in determining the full NHase cluster, classical Southern blotting will be performed to determine the full sequence.

Conclusion

Despite the unfavourable growth characteristics, *N. alkaliphilus* is a stable and versatile nitrile hydrating biocatalyst with a high activity and with only a very limited amount of amidase activity. The cells can be successfully applied in the production of acrylamide and adipamide. The application of the NHase from *N. alkaliphilus* in the production of acrylamide is further discussed in Chapter 5. Furthermore, the whole cells show a reasonable regioselectivity in the hydration of adiponitrile, which might be interesting to investigate in more detail and on a broader substrate scope in the future.

In order to investigate the enantioselectivity of the NHase from *N. alkaliphilus* in Chapter 4, additional (enantioselective) enzyme activities had to be removed from the crude cell-free extract of *N. alkaliphilus.* Cell-free NHase without traces of amidase and esterase activity was prepared by a 7.3-fold purification of the crude cell-free extract using ammonium sulfate fractionation and an anion exchange purification step. The purified enzyme could be efficiently stored as an ammonium sulfate precipitate at 4 ^{o}C until further use.

The α- and β-subunits of the NHase from *N. alkaliphilus* had typical molecular weights around 26 and 28 kDa. At the moment of writing, the whole sequence of the NHase genes and its “activator” are not obtained yet. However, approximately 75 % of the α-subunit is identified. This part of the α-subunit is very similar to other NHase α-subunits and contains the typical Co-type NHase signature C**T**LCSC. Obtaining the complete gene sequence for the α- and β-subunits of this NHase as well as for its activator protein and subsequent expression in *E. coli* may circumvent the problem with the *N. alkaliphilus* growth rate in the future.

Experimental

For a detailed description of the culture conditions for *N. alkaliphilus* refer to Chapter 2. Cultures were stored at -80 °C as a dense cell suspension in 0.5 M sodium carbonate buffer of pH 9.

Chemicals

All chemicals used were purchased with the highest possible purity from Sigma-Aldrich, Fluka, or Acros and used in the experiments without any additional purification. Aromatic and aliphatic α,α-disubstituted cyanohydrin acetates were produced as described previously.[23] Aromatic and aliphatic 2-azidonitriles were a gift from the Institute of Chemical and Engineering Sciences (ICES) Singapore.

Analytical methods

Protein concentrations were determined by using the Bradford assay and/or the Lowry method.[24,25] The ammonium concentration was determined by the phenol-hypochlorite method according to Weatherburn.[26]

The concentration of nitriles, amides, carboxylic acids, and ketones during the reactions was followed by high performance liquid chromatography (HPLC). All compounds were separated by using a Merck Chromolith SpeedROD RP-18e column (50 x 4.6 mm), except for acetonitrile, acetamide, and acetic acid. Acrylonitrile, methacrylonitrile, butyronitrile, iBN, pentanenitrile, 3-cyanopyridine, adiponitrile and their corresponding amides and acids were separated by using an eluent of H_2O-AcN 99 : 1 (v/v) containing acetic acid (0.1 %, v/v). Benzonitrile, phenylacetonitrile, hexanenitrile, 2-azidopentanenitrile, aromatic and aliphatic α,α-disubstituted cyanohydrin acetates and their corresponding amides, acids and ketones were separated by using an eluent of H_2O-AcN 90 : 10 (v/v) containing TFA (0.1 %, v/v). 2-Phenylpropionitrile, 2-azidophenylpropionitrile and their corresponding amides and acids were separated using an eluent of H_2O-AcN 80 : 20 (v/v)

containing TFA (0.1 %, v/v). Propionitrile and its corresponding amide and acid were separated by using an eluent of H_2O and acetic acid (0.1 %, v/v). All separations on the SpeedROD were carried out at 21 °C and with a flow rate of 1 mL·min^{-1}. Acetonitrile, acetamide, and acetic acid were separated by using a Phenomenex Rezex ROA-organic acid H^+ column (300 x 7.80 mm; 8 µm) with an eluent consisting of H_2O with 0.1 %, v/v TFA (0.6 mL min^{-1}) and a column temperature of 60 °C. The aliphatic nitriles were detected by using a Shimadzu RID 10A refractive index detector. All other compounds were detected by using a Shimadzu SPD-10A VP UV-VIS detector. A wavelength of 210 nm was used to detect all aliphatic amides, acids and ketones, and a wavelength of 230 nm was used to detect all aromatic amides, acids and ketones.

Biotransformations

Reactions were carried out in Eppendorf tubes or in 10 mL glass vials on a ThermoTWISTER comfort of QUANTIFOIL Instruments. For reactions at pH 7-9 a 0.01 M Tris-HCl buffer was used, while for reactions at pH 4-6 a 0.01 M citrate buffer was applied and for experiments at pH 10 - 12 a 0.01 M phosphate buffer. To 1 - 10 mL of buffer a certain amount of pure nitrile was added either directly from the source liquid or as a stock solution in MeOH and the reaction mixture was shaken horizontally (700 rpm) at the desired temperature. The reaction was initiated by the addition of cell suspension with a known protein concentration (20 to 25 mg·mL^{-1} protein). Regular samples were taken and quenched in a 1 M HCl solution. After centrifugation of the denatured protein (13000 rpm, RT, 15 min.), the supernatant was either injected directly on HPLC or was diluted first with MilliQ water when necessary.

Purification

Cell disruption

Frozen dense cell suspension was allowed to melt slowly on ice. To 4 mL of this dense cell suspension 36 mL buffer (0.01 M Tris-HCl pH8) was added and the

resulting mixture was vortexed until a homogenous suspension was obtained. Before cell disruption a small amount of RNase, DNase and magnesium chloride were added to the suspension to prevent the formation of a dense and viscous cell-free extract (due to DNA release from the lysed cells). Disruption at a pressure of 2 kbar (IKS Lab Equipment Constant Cell Disruption System) gave a liquid non-viscous cell free extract. Cell debris and remaining whole cells were spun down using a Sorvall RC-5B refrigerated superspeed centrifuge (18000 rpm, 4 ^{o}C, 30 min.). Crude cell free extract was stored on ice without additions until further use.

Ammonium sulfate cut

To crude cell-free extract (35 mL), a saturated ammonium sulfate solution (pH 8, 24.5 mL) was slowly added (41 % saturation). The protein was allowed to precipitate (20 min., 4 ^{o}C) and the suspension was subsequently centrifuged (12000 rpm, 4 ^{o}C, 20 min.), after which the formed protein pellet was discarded. Saturated ammonium sulfate (pH 8, 45.5 mL) was then added to the supernatant (67 % saturation) and after precipitation (20 min., 4 ^{o}C) the suspended protein was centrifuged (12000 rpm, 4 ^{o}C, 20 min.).

Anion exchange chromatography

The pellet of precipitated protein containing the NHase was redissolved in 10.5 mL of buffer A (40 mM HEPES-NaOH buffer of pH 7.3 containing 40 mM hexanoic acid) and the resulting extract was filtered (0.45 µm filter). The semi-purified extract (10 mL) was applied to a DEAE (diethylaminoethyl) Sepharose Fast Flow (GE Healthcare) column of 28 mL (GE healthcare XK16 column) at 4 mL·min^{-1} under the control of a Shimadzu LC-20AT FPLC. The column was equilibrated with buffer A and the same buffer was used to wash the column until no protein was detected in the flowthrough. The enzyme was eluted with a linear gradient of NaCl (0 - 1 M) in buffer A at a flowrate of 5 mL·min^{-1}. Fractions of 10 mL were collected and the protein in these fractions was detected using a Shimadzu SPD-M20A UV-VIS detector at 280 nm. The active fractions (36 - 41) eluted at 330 - 350 mM of NaCl and were pooled and concentrated using a 10 kDa centrifuge filter device.

SDS-PAGE

SDS-PAGE was performed on a Criterion XT system of BioRad using Criterion 4-12 % Bis-Tris XT Precast Gels. To a fraction sample (60 µL) 4x XT sample buffer was added (20 µL) as well as 20x diluted XT reducing agent (4 µL), after which the proteins were denatured by incubating at high temperature (10 min., 99 °C). The samples were applied to the gel together with Precision Plus Protein unstained standards (BioRad). The gel was run for 45 minutes at a current of 200 V. Proteins were visualised by staining with SimplyBlue SafeStain (Invitrogen).

Activity tests

NHase: To a solution of 1000 µL buffer (0.01 M Tris-HCl pH 8) and 10 µL hexanenitrile (80 mM), 10 µL of fraction sample was added. The reaction was allowed to continue for 15 min. (700 rpm, 21 °C), after which a reaction sample was quenched with 1 M HCl. After centrifugation of the denatured protein, the sample was directly injected on HPLC to determine the amount of hexaneamide formed.

Amidase: To 1000 µL of a stock solution of hexaneamide (10 mM in 0.01 M Tris-HCl pH 8), 50 µL of fraction sample was added. The reaction was carried out for 2 h. (700 rpm, 21 °C), after which a reaction sample was quenched with 1 M HCl. After centrifugation of the denatured protein, the sample was injected directly on HPLC to determine the amount of hexanoic acid formed.

Sequence determination NHase gene

Chromosomal DNA of *N. alkaliphilus* was isolated using the NucleoSpin Tissue-kit (Macherey-Nagel). A part of the gene cluster was picked up by PCR with degenerate primers on two conserved regions in the α-subunit of all NHases as described before.[20,21] Different concentrations and combinations of forward and backward primers were used (Table 3.5) until a visible band of approximately the right size appeared on an agarose gel. PCR was performed using 2 $ng \cdot \mu l^{-1}$ template DNA, 4 µM primer, and 0.02 $U \cdot \mu l^{-1}$ Phusion High Fidelity DNA polymerase (Finnzymes) in a step-down PCR protocol: 10 min 95 °C, 7x(45 sec 95 °C, 45 sec 50-

60 °C, 30 sec 72 °C), 20x(45 sec 95 °C, 45 sec 50-60 °C -0.5 °C/cycle, 30 sec 72 °C), 9x(45 sec 95 °C, 45 sec 40-50 °C, 30 sec 72 °C), 10 min 72 °C. This was followed by incubating for 15 min at 72 °C with *Taq* polymerase (Fermentas) to add an extra A for subsequent cloning. PCR products were cloned into pCR2.1-TOPO (Invitrogen).

Table 3.5 All PCR and sequencing primers used in this chapter

Name (forward/reverse)	Sequence (5' → 3')	Source
Degenerate PCR		
NHblockB (f)	GCACCCTGTGCTCCTGCWMNSCNTGGCC	[21]
NHblockB (r)	TTCCTGGGACCAGCCTTCNGTNCCNKC	[21]
NHBlA (f)	GGCGCTAAAGTTGTTGCNMRRGCNTGG	[20]
NHBlB (f)	GGCCGGTCCTGGGNYTSCCNCC	[20]
NHBlC (r)	GCGCATTTCGGCGSWNSWRTCCCA	[20]
Primer walking		
2For (f)	CAACTGGTACAAGTACCCCG	This Chapter
2Rev (r)	GACGCGGATCTCAACCGA	This Chapter
2Rev2 (r)	TTCGCGCACGGCCTTG	This Chapter
Sequencing		
2Seq1 (f)	CAAGGCCGTGCGCGAA	This Chapter
2Seq3 (f)	CTTTCGCTACTCATGCCTAC	This Chapter
2Seq2 (r)	AGGTCGAGGCCGAAGTC	This Chapter
2Seq2A (r)	CGGGGTACTTGTACCAGTTG	This Chapter
2Seq4 (r)	GTCATGATGCCCTTCTCG	This Chapter

The cloned inserts were sequenced (BaseClear) and from this sequence gene walking primers were designed to obtain the rest of the gene cluster. This primer walking was carried out according to the two-step gene walking method,[22] using the following PCR protocol: 10 min 98 °C, 30x(30 sec 95 °C, 30 sec 50-60 °C, 3 min 72 °C), 1x(30 sec 95 °C, 30 sec 40 °C, 4 min 72 °C), 30x(30 sec 95 °C, 30 sec 50-60 °C, 190 sec 72 °C), 10 min 72 °C. The PCR products were directly sequenced using a second internal sequencing primer. New primers were developed upon further reading of the gene (Figure 3.7). Sequences of the primers can be found in Table 3.5.

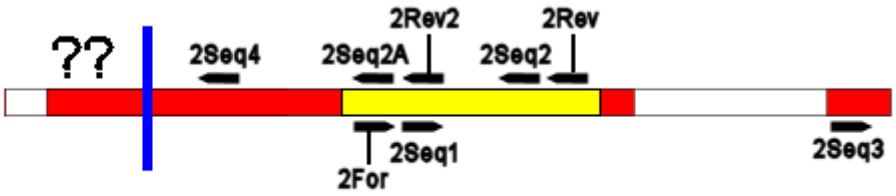

Figure 3.7 A schematic representation of the used primers for the gene walking procedure. Red = open reading frame, yellow = degenerate PCR product.

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4

Probing the Enantioselectivity of a Novel Group of Purified Nitrile Hydratases

Abstract

In this study the purified cell-free Co-containing nitrile hydratases (NHases, EC 4.2.1.84) from *Rhodopseudomonas palustris* HaA2 (HaA2), *Rhodopseudomonas palustris* CGA009 (009), *Sinorhizobium meliloti* 1021 (1021) and *Nitriliruptor alkaliphilus* (iso2), as well as the Fe-containing NHase from *Rhodococcus erythropolis* AJ270 (AJ270) were probed for their enantioselectivity on a group of different chiral nitriles. It was found that the presence of an alkyl group on the α-position of 2-phenylacetonitrile was vital to induce a good enantioselectivity in the Co-containing NHases. Enantiomeric ratios of > 100 were found for the NHases from HaA2 and CGA009 on 2-phenylpropionitrile. In contrast, the Fe-containing NHase was practically aselective for all the different α-phenylacetonitriles.

In general, at least one bulky group in close proximity to the α-position of the chiral nitriles seemed to be necessary for enantioselectivity with all NHases tested and tertiary nitriles were only reluctantly accepted and showed no apparent selectivity. Substituents on the aromatic ring had a positive influence on the enantioselectivity of the AJ270 and iso2 NHases, but decreased the selectivity of the other enzymes. Enantiomeric ratios of 80 and > 100 for AJ270 and iso2, respectively, were found for naproxennitrile and 3-(1-cyanoethyl)benzoic acid was hydrated to the corresponding amide by iso2 with an enantiomeric ratio of > 100.

Introduction

Nitriles are organic compounds widely used in industry as intermediates and building blocks, as well as organic solvents. Nitrile hydrolysing enzymes can be used to produce a wide spectrum of higher value carboxylic amides and acids from the nitrile intermediates. Besides a high yield and a high chemoselectivity because of the mild reaction conditions, enzymatic nitrile hydrolysis also offers the possible advantage of regio-, and enantioselectivity.[1] Two different pathways for the enzymatic hydrolysis of nitriles to the corresponding carboxylic acids exist in Nature.[2] A nitrilase (EC 3.5.5.1) catalyses the direct hydrolysis of a nitrile into the corresponding carboxylic acid and ammonium ion in the first pathway. In the second pathway a nitrile hydratase (NHase, EC 4.2.1.84) catalyses the hydration of a nitrile to the carboxylic amide and subsequently this amide can be hydrolysed into the carboxylic acid and ammonium ion by an amidase (EC 3.5.1.4).

In contrast to nitrilases, early experiments suggested that nitrile hydratases are relatively unspecific with respect to the chirality of the nitrile substrate and that any enantiodiscrimination occurs during the hydrolysis of the intermediate amide by the amidase.[3] However, more complex observations obtained from experiments with whole cells on arylaliphatic nitriles could be explained by the possible existence of enantioselective NHases.[4,5] The first enantioselective NHases were purified from *Pseudomonas putida* strain 5B,[6] *Agrobacterium tumefaciens* strain d3,[7,8] and *Rhodococcus equi* strain A4.[9] The enantioselective NHase from *Pseudomonas putida* was expressed successfully in *E. coli*[10] as well as in *P. pastoris.*[11] All of these purified enzymes show mainly a preference for *(S)*-2-arylpropionitriles and *(S)*-2-arylbutyronitriles. Optically active *(S)*-2-arylpropionamides can be useful as precursors for non-steroidal anti-inflammatory drugs like ibuprofen and naproxen, since the activities have been shown to be much higher for the *(S)*-enantiomers of these medicine.

Recently, the enantioselective NHase from *Rhodococcus erythropolis* strain AJ270 was purified[12] and expressed in *E. coli.*[13] This NHase shows enantioselectivity in the hydration of several racemic *trans*- and *cis*-2-arylcyclopropanecarbonitriles. A

recent patent and paper describe the enantioselectivity and heterologous expression of the NHases from *Raoultella terrigena*, *Pantoea* sp., *Brevibacterium linens*, and *Klebsiella oxytoca.*[14,15] Besides the preference of these NHases for the *(S)*-enantiomer of 2-phenylpropionitrile, they also show a preference for the *(S)*-enantiomers of mandelonitrile and phenylglycine nitrile, although to a lesser extent. BioVerdant developed a process for the production of levitiracetam in which a NHase, modified using directed evolution, is used to produce *(S)*-2-(2-pyrrolidon-1-yl)-butyramide from the racemic nitrile.[16]

Despite an increase in reports in literature describing enantioselective NHases in the recent years, the scope of substrates tested is still narrow and often the data available is not sufficient to calculate the enantiomeric ratio (*E*) values. In this study five purified NHases from different bacterial species are probed for their enantioselectivity on a group of twelve different chiral nitriles (Figure 4.1).

Results and discussion

Purified NHases

In order to accurately determine the enantioselectivity of the NHases, it is necessary to work with the cell-free purified enzymes. The whole cells or crude cell-free extracts can contain a variety of enzymes like nitrilases, amidases, and esterases that can have an influence on the enantiomeric excess (*ee*) of the nitrile substrate as well as the amide product. The NHases used in this study (Table 4.1) were purified by polyhistidine tag (His-tag) affinity chromatography following their expression in *E. coli* or by anion exchange chromatography after induction in the original host.

Table 4.1 The five NHases probed for their enantioselectivity

Organism	Metal	NCBI[a] sequence reference
Rhodococcus erythropolis AJ270[b] (AJ270)[12,13,17]	Fe (III)	α: CAC08205, β: CAC08206
Rhodopseudomonas palustris HaA2[b] (HaA2)	Co (III)	α: YP_486317, β: YP_486318
Rhodopseudomonas palustris CGA009[b] (009)	Co (III)	α: NP_948148, β: NP_948149
Sinorhizobium meliloti 1021[b] (1021)	Co (III)	α: NP_386211, β: NP_386212
Nitriliruptor alkaliphilus[c] (iso2)[18]	Co (III)	Chapter 3

[a.] National Center for Biotechnology Information

[b.] After overexpression in *E. coli* these NHases were purified by His-tag affinity chromatography. The purified cell-free extracts were obtained from Nzomics.

[c.] All amidase and esterase activity was removed by partial purification using anion exchange chromatography (Chapter 3).

All purified cell-free extracts were stored at 4 ^{o}C as their ammonium sulfate precipitate in the presence of 30-40 mM butyric acid at pH 8. Under these conditions, activity loss of all the NHases was negligible over a period of at least 6 months and the extracts could be easily used without the interference of glycerol or the need for thawing and freezing.

Non-chiral nitriles

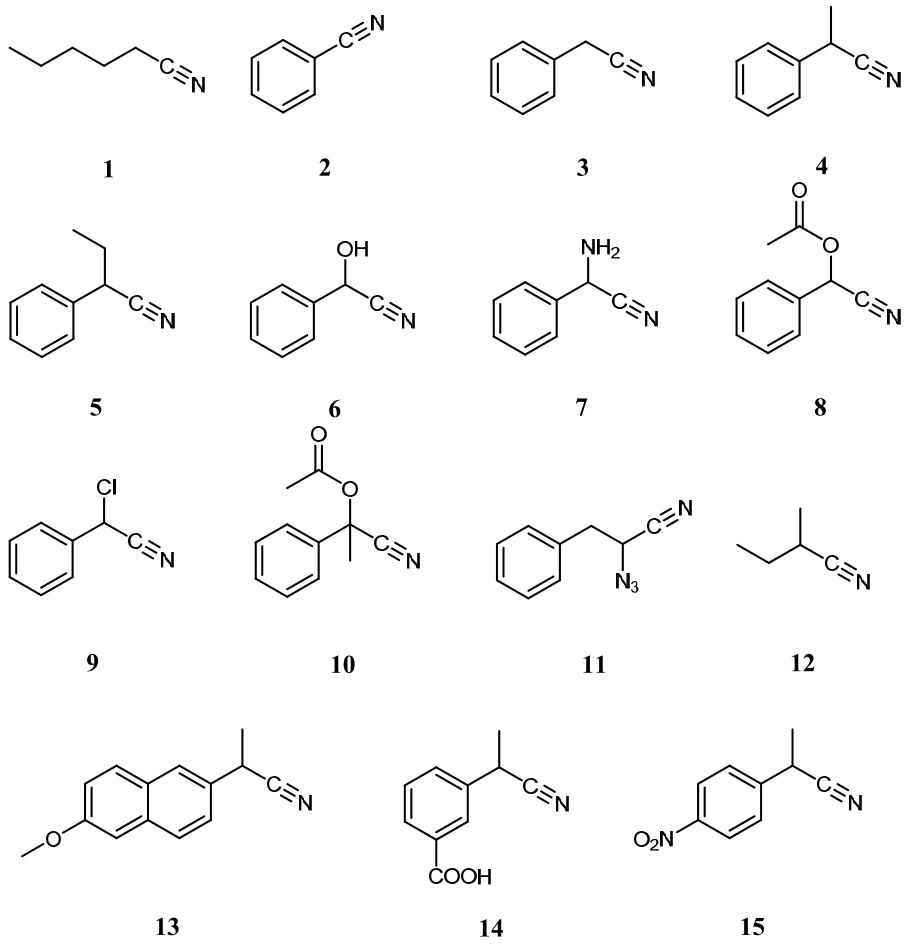


Figure 4.1 The achiral and chiral nitriles which were subjected to biocatalytic hydration by NHases

Because of the differences in the methods of preparation for the purified cell-free extracts of the NHases obtained from Nzomics (AJ270, HaA2, 009, and 1021) and the NHase from *Nitriliruptor alkaliphilus* (iso2), it would not have been accurate to directly compare these enzyme activities and hence relative activities were used. Although the enzyme preparations from Nzomics had a higher purity than the partially purified iso2 enzyme, the activity of the latter was substantially higher. This difference in activity could have been caused by a higher inherent activity of the NHase from iso2. It is however much more likely to be caused by the fact that the Nzomics enzymes were expressed in a heterologous host and that a His-tag was added to these enzymes. The His-tag purification with imidazole may also have had a negative effect on the enzyme activity.

Table 4.2 The relative activities and enantiomeric ratios (*E*) of the biocatalytic hydration of a group of different nitriles using five different NHases.

	Relative activity (%)[a,b]					*E*[c,d] (-)				
Substrate	AJ270	HaA2	009	1021	iso2	AJ270	HaA2	009	1021	iso2
1	100	100	100	100	100	-	-	-	-	-
2	2.6	110	177	131	60.7	-	-	-	-	-
3	11	1.0	0.91	1.0	1.7	-	-	-	-	-
4	0.69	6.0	10	2.4	28	1	>100	>100	18	49
5	$2*10^{-3}$	0.030	0.045	0.028	0.10	7	53	95	5	37
6[e]	0.030	0.40	0.32	0.14	0.22	2	12	11	10	10
7[e]	0.011	1.2	0.82	0.48	0.014	2	5	4	4	7
8[f]	0.14	0.85	0.77	0.41	0.092	4	7	6	5	1(R)
9[g]	2.5	19	18	52	69	2.5(R)	6.6	6.6	1.8	2.1(R)
10[g]	$8*10^{-4}$	$3*10^{-3}$	$3*10^{-3}$	$6*10^{-3}$	$6*10^{-5}$	1	1(R)	1	3(R)	2
11	1.59	60	59	100	26	1.9[h]	2.1[h]	1.7[h]	3.3[h]	2.7[h]
12	67	34	25	28	5.4	1.1	1.4	1.4	1.8	1.3
13	0.23	0.018	0.012	0.013	0.047	80	8.2	8.1	8.0(R)	>100
14	0.016	75	68	38	0.020	6.5[h]	32[h]	34[h]	11[h]	>100[h]
15	0.58	0.30	0.36	0.69	0.45	8.7[h]	21[h]	17[h]	12[h]	15[h]

[a.] The activity of the NHases on hexanenitrile was set to 100 %. These activities are 189, 20, 22, 29, and 358 µmol·min^{-1}·mg^{-1} respectively for AJ270, HaA2, 009, 1021, and iso2.

[b.] Unless stated otherwise, the reactions are carried out at pH 8 and 21 °C.

[c.] $$E = \frac{\ln\left[1-\xi\left(1+ee_P\right)\right]}{\ln\left[1-\xi\left(1-ee_P\right)\right]}$$, when the enantiomeric excess of the product is used (ee_P)[19]

$$E = \frac{\ln\left[\left(1-\xi\right)\left(1-ee_S\right)\right]}{\ln\left[\left(1-\xi\right)\left(1+ee_S\right)\right]}$$, when the enantiomeric excess of the substrate is used (ee_S)[19]

The *E*-value is the average of at least 4 *E*-values calculated at different points of conversion.

[d.] Unless stated otherwise the NHases are *(S)*-selective

[e.] Reactions carried out at pH 5 and 5 °C

[f.] Reactions carried out at pH 7 and 21 °C

[g.] Reactions carried out at pH 6 and 21 °C

[h.] Enantiopure standards to determine the enantiopreference of the enzymes for these substrates were not available. All enzymes produced the same enantiomer.

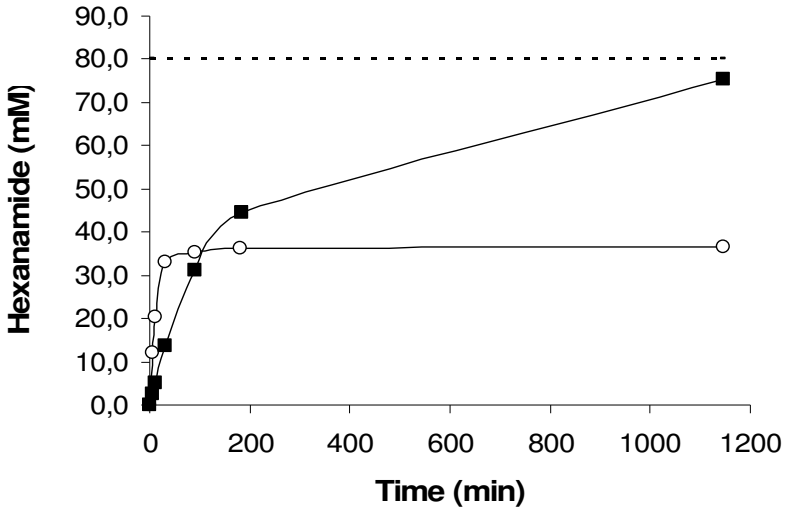


Figure 4.2 The hydration of hexanenitrile (80 mM) using 12.8 mg·L^{-1} AJ270 (○) and 23.6 mg·L^{-1} HaA2 (■) at pH 8 and 21°C.

Among the Nzomic NHases, the Fe-containing AJ270 was most active in hydrating hexanenitrile (**1**) to the corresponding amide (Table 4.2). However, this Fe-containing NHase was less stable than the Co-containing NHases (Figure 4.2). The hydration of **1** using AJ270 halted at a concentration of approximately 35 mM of hexanamide. After the addition of fresh AJ270 the reaction continued, indicating that the enzyme deactivated during the course of the reaction. This relative instability was also observed during the hydration of other substrates. The higher stability of Co-containing NHases was demonstrated before and could be ascribed to the formation of more stable dimers because of the existence of an extra helix in the β-subunit that interacts with the α-subunit.[20,21]

The differences in hydration activity on benzonitrile (**2**) and phenylacetonitrile (**3**) between the Fe- and Co-containing enzymes were remarkable. The Fe-containing NHase clearly preferred the arylaliphatic substrate, while the Co-containing NHases preferred the aromatic one. However, this trend did not apply to the other arylaliphatic substrates.

α-Substituted phenylacetonitriles

The Co-containing NHases from both *Rhodopseudomonas palustris* organisms (HaA2 and 009) showed a very high *(S)*-selectivity for 2-phenylpropionitrile (**4**, Table 4.2, Figure 4.3 a). The Co-containing NHases from *Sinorhizobium meliloti* and *Nitriliruptor alkaliphilus* (1021 and iso2) also converted **4** with a preference for the *(S)*-enantiomer, albeit with a lower selectivity (Figure 4.3 b). The Fe-containing NHase from *Rhodococcus erythropolis* (AJ270) was aselective for this substrate. *E*-values of higher than 100 for **4** have so far only been reported for the NHase of *Agrobacterium tumefaciens* d3.[1,7]

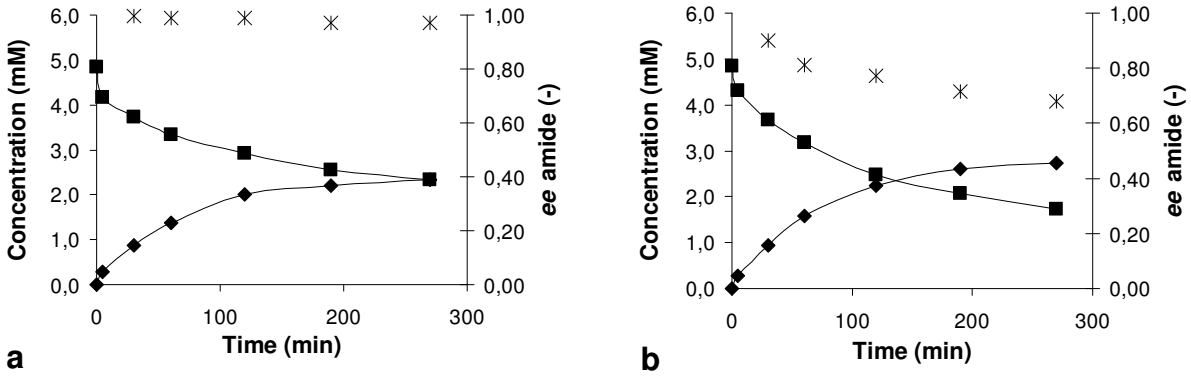
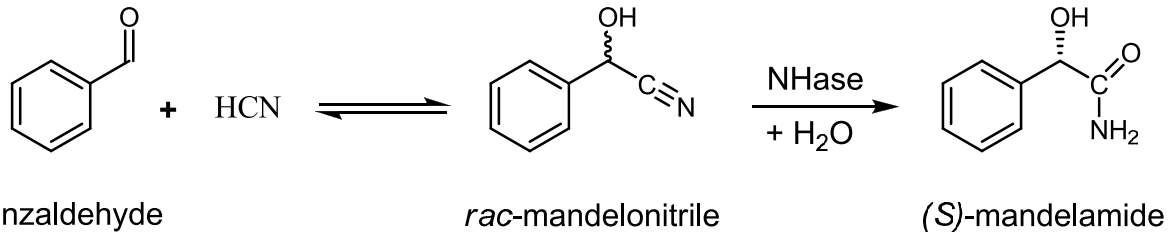


Figure 4.3 The conversion of (**4**) into the corresponding amide by 009 (**a**) and 1021 (**b**) at pH 8 and 21°C, nitrile (■), amide (♦), and *ee* of the amide product (x).

2-Phenylbutyronitrile (**5**) proved to be a challenging substrate for the NHases. The activities of all the NHases on this substrate were low (2-3 orders of magnitude lower than with **4**). The enantioselectivities for **5** followed the same trend as for **4** although in all cases, except for AJ270, the *E*-values were lower. The NHase from *Agrobacterium tumefaciens* d3 showed a similar dip in enantioselectivity when the methyl group on the alpha position is replaced by an ethyl group.[1,7] AJ270 demonstrated low to no selectivity for both **4** and **5**. The Fe-containing NHase from *Rhodococcus equi* A4 was also found to be aselective to these substrates.[9]

The biocatalytic hydration of mandelonitrile (**6**) had to be carried out at low pH and low temperature in order to completely suppress its chemical decomposition. The chemical decomposition reaction of **6** into benzaldehyde and HCN is an

equilibrium resulting in the racemisation of the remaining nitrile substrate and therefore complicating the calculation of the *E*-values. The introduction of a hydroxyl group on the α-position of phenylacetonitrile instead of an alkyl group reduced the enantioselectivity of the Co-containing NHases to an *E* of 10-12 (Table 4.2).



Scheme 4.1 Dynamic kinetic resolution of *rac*-mandelonitrile. Racemisation takes place through the reversible decomposition of mandelonitrile into benzaldehyde and cyanide.

When the racemisation rate of the reversible decomposition reaction of mandelonitrile is high enough compared to the reaction rate of the enzymatic nitrile hydration, the combined process becomes a dynamic kinetic resolution (DKR, Scheme 4.1). A product yield of 100% is theoretically possible in this case and the *ee* of the product will remain constant during the reaction. The main drawback of using the NHases in this DKR, besides the low *E*-values for **6**, was the sensitivity of these enzymes to cyanide.

All of the Co-containing NHases proved extremely sensitive to the cyanide that was released during the racemisation of **6** at pH 7 and 21 °C. Only small amounts of mandelamide can be produced under these conditions (Figure 4.4 b, Table 4.3). The Fe-containing NHase from AJ270 showed a remarkably higher stability and activity in the presence of cyanide (Figure 4.4 a, Table 4.3). Unfortunately, as was demonstrated with **4** and **5**, this enzyme was practically aselective for **6**. The Fe-containing enzyme loses a lot of its activity and stability when the pH is decreased from 7 to 5 (Figure 4.4 c, Table 4.3). However, the negative effect of cyanide on the activity of the Co-enzymes was decreased by carrying out the reaction at low pH and low temperature (Figure 4.4 d, Table 4.3).

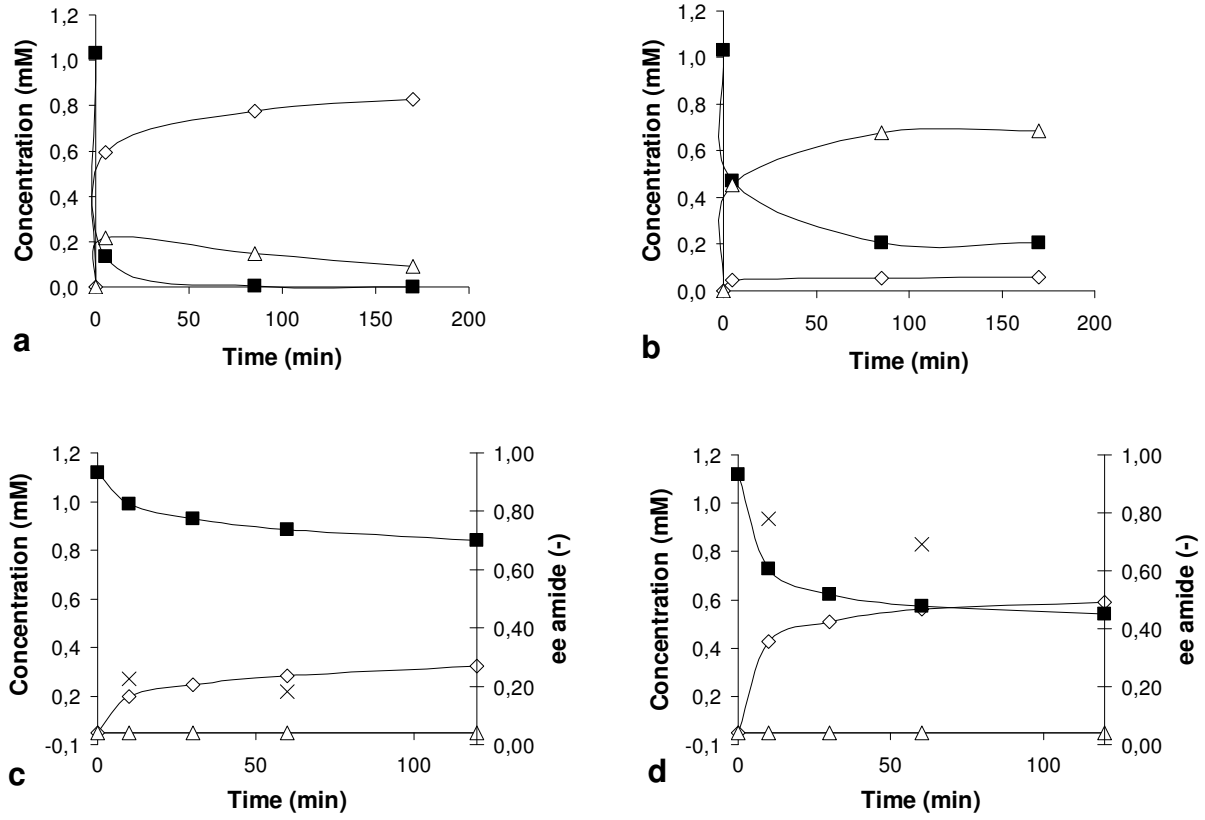


Figure 4.4 The conversion of **6** into the corresponding amide by AJ270 (**a**, **c**) and HaA2 (**b**, **d**) under racemisation conditions (pH 7 and 21 °C, **a, b**) and under acidic conditions with low temperature (pH 5 and 5 °C, **c, d**), nitrile (■), amide (◊), benzaldehyde (Δ), and *ee* of the amide product (x).

The problem of the deactivation of NHases by cyanide was effectively avoided in a patent of Tamura.[22] In this patent Tamura describes a method for producing *(S)*-mandelamide by maintaining the cyanohydrin concentration at a definite and low level by using an automatic cyanohydrin supplier connected to a cyanide detector. It was possible to produce *(S)*-mandelamide from **6** up to a concentration of 1522 mM in 22 hours with an *ee* of 95% at 98.7% conversion using a whole cell *Rhodococcus sp.* HT40-6.[22] A constant *ee* of 95% would imply that the NHase from *Rhodococcus sp.* HT40-6 had an *E*-value of 40 for **6**.

Table 4.3 Activities of the NHases in the hydration of **6** under different reaction conditions.

	protein concentration (mg·mL^{-1})			Activity (µmol·min^{-1}·mg^{-1})		
NHase	pH 7, 21 °C	pH 5, 21 °C	pH 5, 5 °C	pH 7, 21 °C	pH 5, 21 °C	pH 5, 5 °C
AJ270	63[a]	63	248[c]	1.88[a]	0.02	0.06[c]
HaA2	117[b]	117	457[d]	0.08[b]	0.11	0.08[d]
CGA009	63	63	248	0.09	0.13	0.07
1021	154	154	600	0.07	0.06	0.04
iso2	n.d.	n.d.	22.3	n.d.	n.d.	0.69

[a.] Figure 4.4 a [b.] Figure 4.4 b [c.] Figure 4.4 c [d.] Figure 4.4 d

n.d. = not determined

For the same reasons as for **6** the hydration reaction of phenylglycinonitrile (**7**) had to be carried out at low pH and low temperature. Unfortunately an amino group on the α-position of phenylacetonitrile had an even more negative effect on the enantioselectivity of the NHases than a hydroxyl group. The decrease of the enantioselectivity of the Co-NHases when an alkyl group is replaced by an amino or hydroxyl group on the α-carbon was observed previously.[14]

α-Acetoxyphenylacetonitrile (**8**) is not sensitive to HCN elimination but the spontaneous hydrolysis of the ester bond is significant at pH >7. Racemisation can also occur via deprotonation of the α-position, although this process is slow in aqueous media at pH <7. When the reaction was carried out at pH 7 with a high enzyme loading, only trace amounts of mandelonitrile and benzaldehyde were detected and no apparent racemisation took place. The presence of the relatively bulky acetate group of the α-position does not improve the enantioselectivity of the NHases (Table 4.2).

2-Chloro-2-phenylacetonitrile (**9**) is very sensitive to racemisation via deprotonation of the α-position. In order to investigate the extent of racemisation, the hydration reaction was first carried out at different pH values with HaA2 (Figure 4.5). The rate of racemisation clearly decreases with decreasing pH. A dynamic kinetic resolution could be performed by using a pH of 9 or by using a pH of 8 with a lower enzyme concentration, since a small decrease in *ee* could still be detected in the experiment at pH 8. Of all the α-substituted phenylacetonitriles, **9** is by far converted with the highest activity, which is probably due to the strong electron withdrawing

effect of the chloride group, which could make the nitrile carbon more susceptible to nucleophilic attack by an activated water molecule.[23] This theory could also explain the low activity of **5**. Interestingly, the reaction rate seemed to increase with decreasing pH. Since there was practically no racemisation at pH 6, the reactions for the calculation of *E* were carried out at this pH.

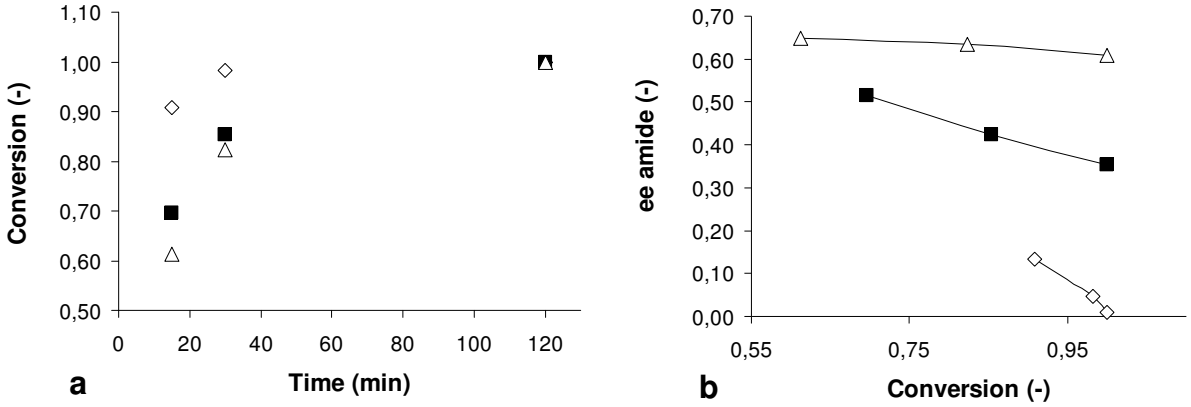


Figure 4.5 The effect of the pH on the conversion (**a**) and the *ee* (**b**) in the hydration of **9** using 451 mg·L^{-1} HaA2 at 21 °C, pH 6 (◊), pH 7 (■) and ph 8 (Δ).

Unfortunately there was no beneficial effect of the chloride substituent on the enantioselectivity of the NHases (Table 4.2). Although selectivity remained bad, AJ270 and iso2 reversed their enantiopreference for this substrate from *(S)* to *(R)*.

Other structurally different nitriles

An additional methyl group at the α-carbon of **8** has a strongly negative effect on the rate of hydration (Table 4.2). Thus, the hydration of 1-cyano-1-phenylethylacetate (**10**) was carried out at pH 6 to avoid the chemical hydrolysis of the acetate group, which would lead to the immediate decomposition of the formed cyanohydrin to phenylacetone and HCN. The tertiairy nitrile group of **10** is probably difficult to access by the NHases because of the steric hindrance of the surrounding groups. Despite the added bulkiness, the additional methyl group also caused a decrease in enantioselectivity.

The azide functionality in 2-azido-3-phenylpropionitrile (**11**) is bulky and charged and was therefore an interesting substituent to test at the α-position for its effect on

the enantioselectivity of the NHases. 2-Azidophenylacetonitrile could not be used because of its extremely low stability. The activities of the NHases on **11** were high (Table 4.2), which is probably caused by the relatively increased distance between the aromatic ring and the α-carbon. The enantioselectivity of the NHases on **11** is low, which is thought to be a combined effect of the nature of the azido group and the added distance between the aromatic ring and the α-carbon.

Replacing the aromatic ring in **4** by an ethyl group causes an almost complete loss of enantioselectivity, indicating that it is necessary to have at least one bulky group attached to the α-position of the nitrile compound to induce enantioselectivity in the Co-containing enzymes.

The relative increase in bulkiness of the aromatic ring in naproxennitrile (**13**) compared to **4** had a positive effect on the enantioselectivity of AJ270 and iso2. This effect was also observed using the Fe-containing NHase from *Rhodococcus equi* A4.[9] In contrast, the Co-containing NHases HaA2, 009, and 1021 lost enantioselectivity when the bulkiness of the aromatic ring was increased, although 1021 switched its enantioselectivity from *(S)* to *(R)*. A switch in enantioselectivity was also observed using the NHase from *Pseudomonas* putida.[1,5,24] The low activity for the hydration of **13** is probably caused by a combination of its bulkiness and the extremely low solubility of this nitrile.

The introduction of a nitro group at the *para*-position of the aromatic ring of **4** reduces the rate as well as the enantioselectivity of the hydration reaction, except in the case of AJ270 (Table 4.2). The introduction of a carboxylic acid group at the *meta*-position of **4**, in contrast, caused some remarkable differences in activity. Both AJ270 and iso2 became substantially less active while the other Co-containing NHases increased their activity. The *E*-values for the hydration of 3-(1-cyanoethyl)benzoic acid (**15**) follow the same trend as for 2-(4-nitrophenyl)propanenitrile (**14**) compared to **4** with the remarkable exception of iso2 which increased in enantioselectivity to an E > 100 (Table 4.2). Organic acids usually act as inhibitors for NHases, which might explain the drop in activity of AJ270 and iso2 with **15** as substrate.

Conclusion

The presence of an alkyl group at the α-position of 2-phenylacetonitrile seemed to be vital to induce a good enantioselectivity in the Co-containing NHases that were screened in this study. The Fe-containing NHase was practically aselective for all α-phenylacetonitriles tested. The NHases needed at least one bulky group close to the α-position of the chiral nitrile for enantioselectivity and tertiary nitriles are only reluctantly accepted and showed no apparent selectivity. Substituents on the aromatic ring had a positive influence on the AJ270 and iso2 NHases but decreased the selectivity of the other enzymes. Since NHases are highly conserved and some crystal structures of Co-NHases are available as well as the crystal structure of AJ270, it might be interesting to use the data in this study in docking studies to possibly identify the amino acid residues that are essential for the enantioselection of these substrates.

The addition of the nitrile in a fed-batch fashion will allow for a higher build-up of the amide product in the reaction and will reduce the negative effect of the nitrile on the stability of the NHases. In an ideal case the enantiopure amide product will precipitate during the reaction, simplifying down stream processing. Solvent engineering will not only improve the solubility of the hydrophobic nitriles but could also possibly improve the enantioselectivity of the NHases. The high sensitivity of these cell-free purified NHases to co-solvents as well as 2-phase systems is however a limiting factor.

Experimental

General

The purified NHases from *Rhodococcus erythropolis* AJ270, *Rhodopseudomonas palustris* HaA2, *Rhodopseudomonas palustris* CGA009, and *Sinorhizobium meliloti* 1021 were a gift from Nzomics.[ξ] The NHase from *Nitriliruptor alkaliphilus*[18] was purified as described previously (Chapter 3). All the purified NHases were stored at 4 °C as their ammonium sulfate precipitate in the presence of 30-40 mM butyric acid at pH 8. Reactions were carried out in Eppendorf tubes using a ThermoTWISTER comfort shaker of QUANTIFOIL Instruments. Protein concentrations were determined using the Bradford assay.[25] For experiments at pH 7-8 a 0.01 M Tris-HCl buffer was used, while for experiments at pH 5-6 a 0.01 M citrate buffer was applied.

Chemicals

Hexanenitrile (98 %, Aldrich), hexanamide (98 %, Aldrich), benzonitrile (≥ 99 %, Fluka), benzamide (99 %, Aldrich), phenylacetonitrile (≥ 99 %, Aldrich), *rac*-2-phenylpropionitrile (96 %, Aldrich), *(S)*-2-phenylpropionic acid (97 %, Acros), 2-phenylbutyronitrile (95 %, Aldrich), *(R)*-2-phenylbutyric acid (99 %, Aldrich), *rac*-mandelonitrile (90%, Fluka), *rac*-mandelamide (97 %, Alfa Aesar), *(R)*-mandelamide (≥ 97%, Aldrich), benzaldehyde (99.5 %, Acros), *rac*-phenylglycinonitrile·HCl (technical grade, Acros), 2-hydroxy-2-phenylpropionamide (Sigma), phenylacetone (99 %, Aldrich), 2-methylbutyronitrile (> 80 %, Lonza quality, Aldrich), *(S)*-2-methylbutyronitrile (98 %, Aldrich), *(S)*-naproxen (98 %, Sigma), 3-(1-cyanoethyl)benzoic acid (98 %, Aldrich), 2-(4-nitrophenyl)propanenitrile (97 %, Aldrich) were used in the experiments as received without additional purification. 1-

[ξ] Nzomics Biocatalysis: www.Nzomics.com

Cyano-1-phenylethylacetate was produced as described previously.[26] Racemic naproxennitrile and racemic naproxenamide were a gift from Andreas Stolz of the Institut für Mikrobiologie der Universität Stuttgart and *(R)*-phenylglycinoamide (> 99 %) was a gift from DSM. *rac*-2-Azido-3-phenylpropionitrile and *rac*-2-azido-3-phenylpropionamide were a gift from the Institute of Chemical and Engineering Sciences (ICES) Singapore.

(S)-2-Phenylpropionamide, *(R)*-2-phenylbutyramide, and *(S)*-naproxenamide were prepared by reacting the corresponding enantiopure acids with $SOCl_2$ at 80 °C for 1 hour. After the evaporation of excess $SOCl_2$, concentrated ammoniumhydroxide solution (25 w % ammonia) was added to the formed acyl chloride. Amide crystals immediately formed on cooling the reaction mixture and the product was purified through recrystallisation in water. In a similar fashion *rac*-2-chloro-2-phenylacetonitrile was prepared by the reaction of *rac*-mandelonitrile with thionylchloride in chloroform and *(R)*-2-Chloro-2-phenylacetamide was synthesised from *(R)*-mandelic acid.[27]

The purification of technical *rac*-phenylglycinonitrile·HCl was carried out by the neutralisation of the hydrochloric acid salt with a 1 M NaOH solution followed by an extraction with dichloromethane. The combined organic layers were acidified with a 1 M HCl solution and the nitrile hydrochloric acid salt was re-extracted in the aqueous phase, which was concentrated in *vacuo*, resulting in pure white *rac*-phenylglycinonitrile·HCl crystals. **Warning:** during the neutralisation of *rac*-phenylglycinonitrile·HCl large amounts of HCN are liberated. This experiment should always be performed in a fume cupboard with a good draught. It is strongly advised to use a well-calibrated HCN detector.

rac-α-acetoxyphenylacetonitrile was synthesised by the acetylation of *rac*-mandelonitrile with acetic anhydride and pyridine in dichloromethane at room temperature. The same procedure was used for the acetylation of *(S)*-mandelonitrile to produce *(S)*-α-acetoxyphenylacetonitrile and for the acetylation of 2-hydroxy-2-phenylpropionamide to produce the amide of cyano-1-phenylethylacetate. *(S)*-mandelonitrile was produced via the enzymatic hydrocyanation of benzaldehyde using the oxynitrilase from *Manihot esculenta*.[28]

Biotransformations

To 1 mL of buffer, a certain amount of nitrile substrate was added. Before the addition of nitrile a stock solution in MeOH was prepared for the nitriles that were difficult to dissolve in water, although the concentration of MeOH in the reaction mixture was kept < 5 v % to prevent rapid deactivation of the NHases. Special care was taken to carry out the reactions at a nitrile concentration where complete dissolution is assured, since incomplete dissolution will result in inaccurate *E*-values.

After taking a sample to determine the exact starting concentration of the reaction, the reaction was commenced by the addition of enzyme directly from the ammonium sulfate stock suspension. When more than 25 µL of enzyme suspension had to be added, the suspension was first centrifuged, after which the pellet was redissolved in the reaction buffer before addition to the reaction. Periodically samples were withdrawn from the reaction and analysed on chiral and non-chiral HPLC as well as on chiral GC.

For reversed-phase HPLC the enzyme in the sample was denatured by the addition of 1 M HCl, after which denatured NHase was spun down (13000 rpm, 5 min). When necessary the sample was diluted with MilliQ and subsequently directly injected on HPLC. For straight-phase HPLC, a reaction sample was added to a mixture of hexane-isopropanol 80:20 (v/v). After adding magnesium sulfate to dry the sample, the sample was centrifuged (13000 rpm, 5 min) and the supernatant was injected on HPLC. The same sample methodology was used for chiral GC but the sample was added to diethylether instead of hexane-isopropanol.

Analyses

Achiral

All achiral analyses were carried out on a 4.6 x 50 Merck Chromolith SpeedROD RP-18e with different eluent compositions containing 0.1 v % TFA as organic modifier at 1 $mL \cdot min^{-1}$ and a column temperature of 21 ^{o}C. Compounds were detected using a Shimadzu SPD-10A VP UV-VIS detector at different wavelengths

and a Shimadzu RID 10A refractive index detector. The following conditions allowed for baseline separation of the mentioned nitriles and their corresponding amides as well as any occurring side products like aldehydes: compound **1** – **3** (H_2O – AcN 90 : 10 (v/v)), compound **4** – **5**, **8** – **9**, **10** – **11**, **14** (H_2O – AcN 20 : 80 (v/v)), compound **6** (H_2O – AcN 97.5 : 2.5 (v/v)), compound **12** (H_2O – AcN 99 : 1 (v/v)), compound **13** (H_2O – MeOH 60 : 40 (v/v)), and compound **15** (H_2O – MeOH 70 : 30 (v/v)). For all compounds a UV detection wavelength of 210 nm was used, except for **2** – **3** (230 nm) and **12** (205 nm).

Chiral

Unless stated otherwise, the compounds were detected using a Waters 486 Tunable Absorbance Detector at 210 nm.

The enantiomers of the amide of **4** were separated on a 250 x 4.6 mm Chiralcel OD column, eluent hexane-isopropanol, 80 : 20 (v/v) containing TFA (0.1 %, v/v) at 0.5 mL min^{-1} and a column temperature of 21 °C. The *(S)*-enantiomer eluted before *(R)*. The same analysis method was used for the amide of **5** (*(S)* elutes before *(R)*) and **13** (*(S)* elutes before *(R)*). The enantiomers of the amide of **14** were separated on the same column using heptane-isopropanol, 90 : 10 (v/v). Although no enantiomerically pure standard was available for this compound, extrapolation of the previous results might indicate that the *(S)*-enantiomer elutes before *(R)*.

The enantiomers of the amide of **6** were separated using a Nucleodex β-OH column, eluent H_2O-MeOH, 90 : 10 (v/v) at 0.7 mL min^{-1} and a column temperature of 21 °C. The compounds were detected using a Shimadzu SPD-10A VP UV-VIS detector at a wavelength of 210 nm. *(R)*-mandelamide eluted from the column after *(S)*-mandelamide.

The enantiomers of **7** and the enantiomers of the amide of **7** were separated on a Daicel Chemical Industries Ltd. 4.6 x 150 mm; 5 µm Crownpak CR (+) column. The eluent was aqeuous $HClO_4$, pH 1, at a flowrate of 0.6 mL/min. The column temperature was 18 °C. In case of both the nitrile and the amide, the *(R)*-enantiomers eluted before the *(S)*. The compounds were detected using a Shimadzu SPD-10A VP UV-VIS detector at a wavelength of 210 nm.

The enantiomers of the amides of **8** and **9** were separated on a 250 x 4.6 mm Daicel ChiralPak AD-H column, eluent hexane-isopropanol, 92 : 8 (v/v) containing TFA (0.1 %, v/v) at 0.5 mL min^{-1} and a column temperature of 21 ^{o}C. In both cases the *(R)*-enantiomer elutes before *(S)*. The same analysis method was used for the separation of the enantiomers of the amide of **10**. In order to determine the elution order of the *(R)*- and *(S)*-amide, a previously described chiral GC method was used first to determine the configuration of the remaining nitrile.[26] Using this method it was found that the *(S)*-amide eluted before the *(R)*-amide. The enantiomers of the amides of **11** and **15** were separated on the same column using hexane-isopropanol, 97 : 3 (v/v) containing TFA (0.1 %, v/v) for the amide of **11** and heptane-isopropanol, 85 : 15 (v/v) containing TFA (0.1 %, v/v) for the amide of **15**.

The enantiomers of **12** were separated using chiral GC on a Chiradex GTA column of 50 m x 0.25 mm, d_f 0.12 µm with an isothermal temperature program of 50 ^{o}C for 10 minutes and a gas flow of 7 mL/min. (R)-**12** elutes before (S)-**12**.

Acknowledgements

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5

Nitrile Hydratase CLEAs: The Immobilisation and Stabilisation of an Industrially Important Enzyme

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Abstract

The successful immobilisation and stabilisation of a nitrile hydratase in the form of a cross-linked enzyme aggregate (CLEA®) is described. CLEAs were prepared by using ammonium sulfate as an aggregation agent followed by cross-linking with glutaraldehyde. The effect of different glutaraldehyde concentrations on the recovery of enzyme activity in the CLEA and enzyme leakage from the CLEA matrix was investigated. Although activity recovery was low (21 %) the CLEA facilitates easy separation and recycling of the nitrile hydratase. It was also found that the nitrile hydratase CLEA had substantially increased storage stability as well as increased operational stability during exposure to high concentrations of acrylamide and acrylonitrile compared to that of the nitrile hydratase in the crude cell-free extract and whole cell formulation.

Introduction

Nitrile hydratase (NHase, EC 4.2.1.84) is an enzyme class that catalyses the hydration of a very broad scope of nitrile compounds into the higher value amides.[1,2] NHases are industrially important enzymes. The best known process in which a NHase is used is the production of acrylamide. Mitsubishi Rayon uses the enzyme from the genetically modified *Rhodococcus rhodochrous* J1 for the production of >100.000 ton/year of acrylamide.[3-6] SNF Floerger licenced the Mitsubishi Rayon process and announced the production of 100.000 ton/year of acrylamide in Europe.[7] Another process is the NHase catalyzed hydration of 3-cyanopyridine into nicotinamide, which is carried out by Lonza at > 3500 ton/year using the same organism.[8]

The enzymatic process for the hydration of acrylonitrile replaced one which employed a heterogeneous copper catalyst and was performed at 120^{o}C. The latter process afforded a product that is contaminated with traces of copper and significant amounts of acrylic acid byproduct which complicates downstream processing. NHases, in contrast, catalyse the hydration reaction at ambient temperature and pH in high selectivity and high yield. This affords a process that is highly energy efficient, environmentally friendly, and safe and a product of high quality such that no further purification is necessary.[5,8,9] Consequently, the pioneering efforts of Mitsubishi Rayon and Lonza are leading to the widespread replacement of abiological hydration of nitriles by greener enzymatic nitrile hydration using NHases.

However, one of the drawbacks of using NHases is that they are remarkably unstable under reaction conditions, especially when the enzyme is used in a cell-free formulation.[10,11] This explains why most enzymatic hydrations are carried out using whole cells or immobilised whole cell formulations[12-14] and not the free (purified) enzyme.[2]

Because of the possible utilisation of products by cells, and because of the presence of other enzymes such as amidases or proteases, the use of (partially) purified cell-free NHases would be preferred above whole cell catalysis. An efficient

immobilisation method for cell-free NHase will make the application of green enzymatic hydration processes even more attractive.

In this study, the immobilisation and stabilisation of a cell-free NHase from the haloalkaliphilic actinobacterium strain *Nitriliruptor alkaliphilus* in the form of a Cross-Linked Enzyme Aggregate (CLEA) is described. This latter method comprises the aggregation of the free enzyme from an aqueous solution using a precipitation agent, followed by cross-linking of the formed aggregates using a bifunctional cross-linker.[15-17]

Parameters of catalyst performance, such as activity retention and storage, recyclability, pH and temperature stability will be assessed. Since the production of acrylamide is currently the major application for NHases in industry, the performance of the NHase CLEA in the hydration of acrylonitrile is compared to that of the NHase in whole-cell and cell-free formulation.

Results and discussion

CLEA preparation

The preparation of a CLEA involves the semi-purification and precipitation of the enzyme using an aggregation agent, followed by cross-linking using a compound with two or more aldehyde groups. Because CLEAs are prepared using the cell-free enzyme, breaking the cell walls of the enzyme host is a necessary first step.

Cell disruption by rapid decompression was effective, leading to a NHase deactivation of only 8.5% by volume.[†] The crude extract was semi-purified via an ammonium sulfate cut. All proteins that precipitated at an ammonium sulfate saturation of 41 % (v/v) were discarded. Of the total NHase activity 3% was lost in the precipitate but 40 wt% of non-catalytic protein was removed in the process.

Ammonium sulfate (67 % v/v, total saturation) was chosen as the aggregation agent for CLEA preparation. Approximately 80% of NHase activity was recovered

[†] Volumetric activity of cell suspension vs. volumetric activity of extract after disruption

after precipitation of all soluble proteins and redissolution of the precipitate in a buffer.‡ Because of the good results obtained with ammonium sulfate it was decided not to investigate other possible precipitation agents.[18]

Subsequently, CLEAs were prepared from the precipitate with different concentrations of glutaraldehyde (Table 5.1). Leakage of enzyme from the CLEA matrix in the first 3 washing steps decreased substantially when the glutaraldehyde concentration during the cross-linking process was higher. This decrease in leakage is ascribed to more intensive cross-linking. The effect of a higher degree of cross-linking was also visually observed after centrifugation of the CLEAs. The CLEA became more solid, and more difficult to resuspend after the first washing and centrifugation steps, as the glutaraldehyde concentration was increased. For the two highest glutaraldehyde concentrations (3.3 and 4.2 wt %) it was even necessary to cut the precipitated CLEA in smaller pieces, since resuspension was impossible. The remaining activity in the CLEA first increased with increasing glutaraldehyde concentration, presumably due to a decrease in initial enzyme leakage. At a glutaraldehyde concentration > 0.6 wt % the remaining activity declined again. This decrease in activity is ascribed to deformation of the enzyme quaternary structure because of more intensive cross-linking.

Table 5.1 Preparation of CLEAs using different concentrations of glutaraldehyde

Glutaraldehyde concentration (wt %)	Protein concentration (mg/mL)	Activity in supernatant after 1st wash (%)[a]	Activity in supernatant after 3rd wash (%)[a]	Remaining activity in CLEA (%)[b,c]
0.3	3.8	4.5	0.7	15
0.6	3.8	2	0.3	23
1.2	3.7	0.02	0	21
2.3	3.5	0.02	0	16
3.3	3.4	0	0	10
4.2	3.2	0	0	4

[a] % of total CLEA activity. [b] Activity of CLEA is tested after 3 washing and centrifugation steps.
[c] Remaining activity is determined by comparison with the total amount of units in 500 µL crude extract

‡ Unless stated otherwise, the buffer used is 0.01 M Tris-HCl buffer of pH 8

For further tests CLEAs were prepared using 1.2 wt % of glutaraldehyde since this was the CLEA preparation with the highest activity of the preparations where no leakage was detected after the third washing step. Recovery of activity in this CLEA was 21 %. Loss of NHase activity during the CLEA preparation, besides initial enzyme leakage and deactivation because of precipitation, can be ascribed to either deformation of the enzyme quaternary structure caused by the fixation during cross-linking or by mass transfer limitation in the CLEA matrix.

Dextranpolyaldehyde with a M_w of 100 – 200 kDa was tried as an alternative cross-linking agent in an attempt to increase CLEA residual activity.[19] In order to prevent hydrolysis of the formed Schiff's base connections in this CLEA, the imine group was reduced to an amine group using $NaBH_4$. Although the residual activity increased to 40-45%, the storage stability and stability during exposure to high concentrations of nitrile and amide were substantially lower (data not shown). The main problem of the CLEA in this form is the difficulty in handling the immobilised enzyme. The CLEA is very amorphous in suspension and has a low density. Obtaining a good dispersion of the CLEA in the reaction mixture is almost impossible and recycling the CLEA is difficult, since it is hard to separate the CLEA by means of centrifugation.

Storage stability

Figure 5.1 compares storage stabilities of the NHase in crude cell-free extract, whole cell and CLEA formulation at 21 ^{o}C in buffer. The NHase activity in the cell-free extract disappeared completely after 17 days. Cells still have 20% residual activity at this point in time, while the CLEA did not lose any activity. Even after 4 months of storage the CLEA was still fully active. Apparently, cross-linking the NHase leads to drastic stabilisation of the enzyme under these storage conditions.

Freeze drying of the CLEA in most cases caused high activity loss (70-80 % of the total activity). Since the stability of the CLEA stored in buffer was excellent, optimisation of the freeze drying process was not pursued any further.

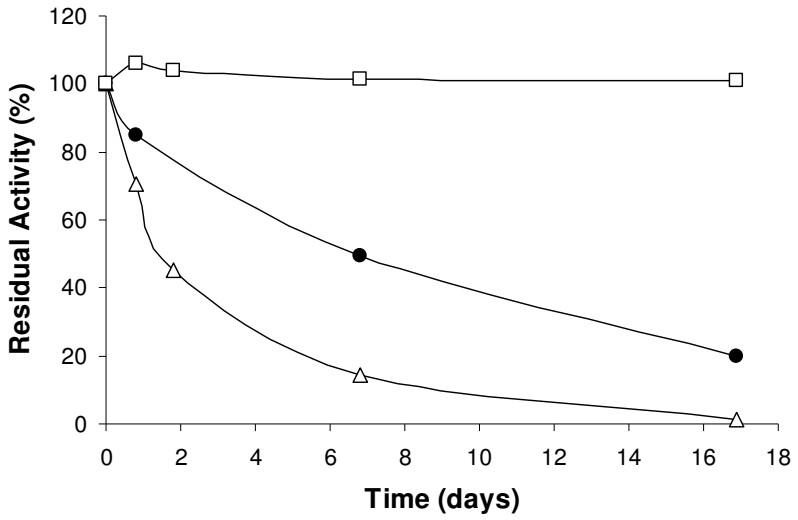

Figure 5.1 Comparison of storage stabilities at 21 °C of crude cell-free extract (Δ), whole cells (●), and CLEA (□) in 0.01 M Tris-HCl buffer of pH 8.

Temperature stability and pH profile

Figure 5.2 shows the residual activities of the NHase in whole cell, cell-free extract and CLEA formulation after 20 minute incubation at different temperatures. Although there seems to be a trend in stability at elevated temperatures of CLEA > whole cell > crude extract, the differences are small. It is well-known that NHases are not stable for a long time at temperatures > 30 °C, except for the NHases that were isolated from thermophilic organisms.[20-23] The low temperature stability of the NHase enzyme is one of the reasons why the production of acrylamide is usually carried out at T < 10 °C. Cross-linking the enzyme clearly does not have a significant effect on the stability of the NHase at elevated temperatures.

The pH profiles (Figure 5.3) of the NHase in whole cell, crude extract and CLEA formulation are largely similar. The NHase in whole cell formulation is less susceptible to pH change than the NHase in the crude extract. This can be caused by the pH regulating capacity of the cells. This effect is most noticeable at pH 11 and 12 where no activity was found for the free enzyme. The CLEA seems to be the least susceptible to pH change. Between pH values of 6-11 the activity differences stay within 20%. The activity range of the CLEA is large, between pH 4 and pH 11. The

better stability of the CLEA during the 20 minute incubation is the main reason for this behaviour.

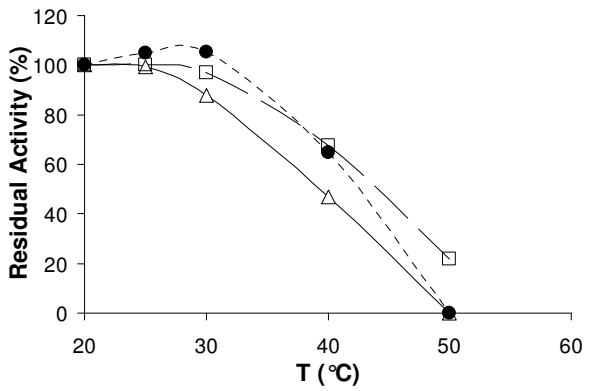

Figure 5.2 Comparison of the temperature stability of NHase in crude cell-free extract (Δ), whole cell (●) and CLEA (□) preparation.

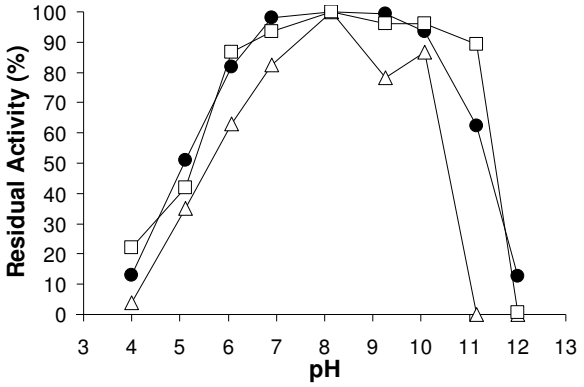

Figure 5.3 Comparison of pH profiles of NHase in crude cell-free extract (Δ), whole cell (●) and CLEA (□) preparation.

Operational stability in acrylamide production

In the production of acrylamide (Figure 5.4), whether it is performed in batch or fed-batch fashion, the NHase is by far most stable in CLEA formulation (Figure 5.5, 5.6 and Table 5.2), especially during exposure to high nitrile and/or amide concentrations. The NHase in cell-free crude extract clearly has the lowest stability. Apparently free NHase is much more sensitive to high acrylamide and acrylonitrile concentrations than NHase contained in cells or cross-linked as a CLEA.

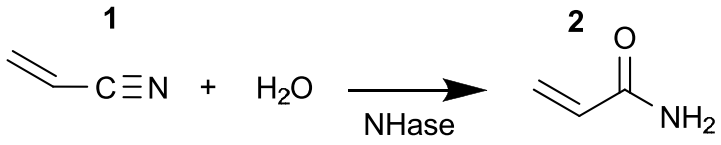

Figure 5.4 Acrylonitrile (1) hydration to acrylamide (2) using a NHase.

One explanation for the increased stability of the CLEA could be that the NHase in this formulation is quite rigidly bound to its neighbours, preventing dissociation of subunits. Dissociation of subunits can lead to lower stability since subunits are usually more susceptible to deactivation factors such as temperature, pH and high concentrations of organic compounds.[24] This effect can also explain the higher

stability of the NHase in a whole cell formulation, since the cells are able to maintain a local high concentration of enzyme which is favourable for the dissociation equilibrium. However, cells have the tendency to lyse during exposure to high concentrations of organics after which the enzyme loses this natural barrier to the environment.

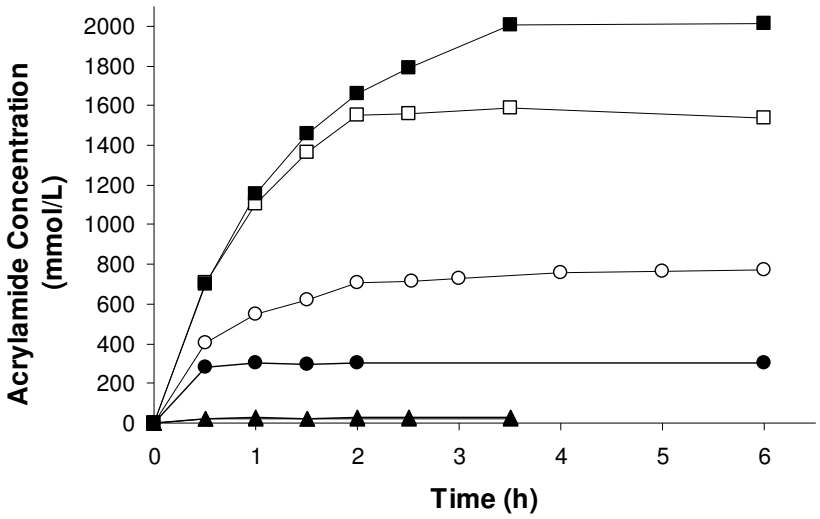

Figure 5.5 Comparison of the stabilities of NHase in crude cell-free extract (Δ, ▲), whole cell (○, ●), and CLEA (□, ■) preparation in batch reactions using acrylonitrile concentrations of 1630 (open symbols) and 1980 (closed symbols) mmol/L.

Another reason for the higher stability of the CLEA could be the existence of concentration gradients inside the CLEA particles. If this is the case, only the outside layer of enzyme in the particle is subjected to high concentrations of amide and nitrile. The outside layer of enzyme can deactivate but will in this way still protect the enzyme that is situated in the inner part of the CLEA particle.

Table 5.2 Effect of starting acrylonitrile concentration on the reaction stability of NHase in different formulations. Residual activities of the CLEA after the batch experiments and during the fed-batch reactions are included.

NHase formulation	Acrylonitrile concentration (% v/v)	Acrylonitrile concentration (mmol/L)	Final conversion (%)	Residual activity (%)
Crude extract	2.9	440	>98	n.d. [a]
	5.7	860	75	
	8.3	1250	8	
	10.7	1630	2[b]	
	13	1980	1[b]	
Whole cell	2.9	440	>98	n.d. [a]
	5.7	860	>98	
	8.3	1250	>98	
	10.7	1630	49[b]	
	13	1980	15[b]	
CLEA	8.3	1250	>98	77
	10.7	1630	>98[b]	65
	13	1980	>98[b]	72
CLEA	Fed-batch cycle #1[c]			68
	Fed-batch cycle #2[c]			53
	Fed-batch cycle #3[c]			27

[a] n.d. = not determined. [b] See also Figure 5.4. [c] See also Figure 5.5.

Another important operational parameter is the recycling stability of the CLEA. Centrifugation and washing of the CLEA after short reactions had practically no effect on the activity[§] (35 cycles were performed) as long as decantation of reaction and washing liquids is performed with care. The particle size distribution of the CLEA is broad and approximately 30-40% of the CLEA is present in fine powder form. The CLEA powder easily resuspends and can be discarded if decantation does not take place carefully.

[§] No activity loss was observed after the 3 initial washing and centrifugation steps

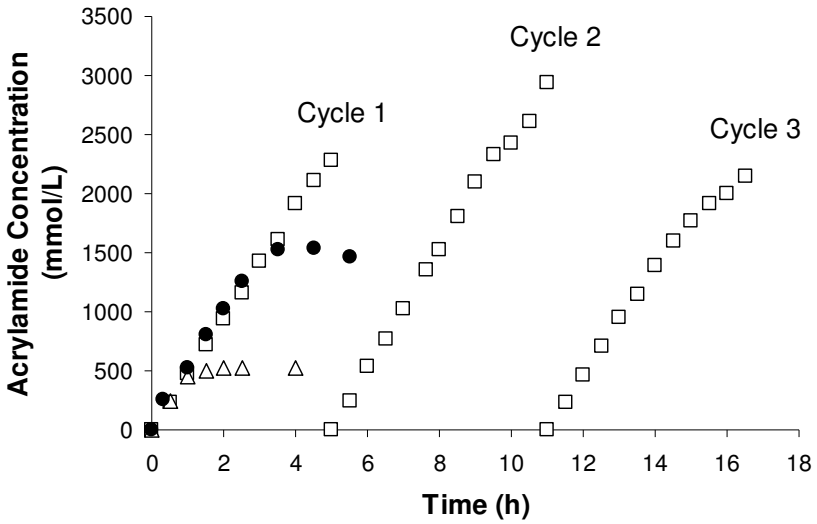

Figure 5.6 Comparison of the stabilities of NHase in crude cell-free extract (Δ), whole cell (●) and CLEA (□) in the fed-batch production of acrylamide.

Conclusion

Although there is room for improvement, the CLEA immobilisation technique can be used to successfully immobilise and stabilise crude cell-free NHase. This is the first time that successful immobilisation of free NHase is reported in the literature. Preparation of the CLEAs starting from the whole cell suspension is simple and straightforward, which makes it a very accessible method of immobilisation. Continuously improving the methods of NHase immobilisation will contribute to more applications of this enzyme as a green nitrile hydration catalyst in industry and therefore will contribute to green chemistry in general.

Experimental

Haloalkaliphilic actinobacterium *Nitriliruptor alkaliphilus*, enriched from soda lake sediments, was grown as described previously with isobutyronitrile as the only carbon, energy and nitrogen source.[25] Freshly grown cells were washed in 0.3 M sodium carbonate/bicarbonate buffer, pH 9 and stored at -80 ^{o}C as a dense

suspension until further use. Protein concentration was measured by using the Lowry method. Hexanenitrile (98 %), hexanamide (98 %) and hexanoic acid (99.5+ %) were purchased from Aldrich. Acrylonitrile (>99.5 %), acrylamide puriss. p.a. standard for GC (>99.8 %) and anhydrous acrylic acid (> 99 %) were purchased from Fluka. A 25 wt % solution of glutaraldehyde in water was purchased from Acros. Deoxyribonuclease 1 and ribonuclease A, both from bovine pancreas, were purchased from Sigma. In all experiments the buffer that was used is 0.01 M Tris-HCl buffer of pH 8 unless stated otherwise.

Cell disruption

Frozen dense cell suspension (protein concentration: 60 mg/mL) was allowed to melt slowly on ice. To 2 mL of this dense cell suspension 3 mL buffer was added and the resulting mixture was vortexed until a homogenous suspension was obtained. Before cell disruption a small amount of RNase, DNase and magnesium chloride were added to the suspension to prevent the formation of a dense and viscous cell-free extract (due to DNA release from the lyzed cells). Disruption at a pressure of 2Kbar (IKS Lab Equipment Constant Cell Disruption System) gave a liquid non-viscous cell free extract. Cell debris and remaining whole cells were spun down using a Sorvall RC-5B refrigerated superspeed centrifuge (18000 rpm, 4 ^{o}C, 30 min.). Cell free extract was stored on ice without additions until further use.

CLEA preparation using glutaraldehyde

An amount of 500 µL of cell free extract (protein concentration: 16.2 mg/mL) was shaken in an Eppendorf tube (500 rpm, 4 ^{o}C, ThermoTWISTER comfort, QUANTIFOIL Instruments). The total amount of units in 500 µL of extract was approximately 100. To the extract, 350 µL of saturated ammonium sulfate (pH adjusted to 8 using NaOH) was slowly added (41 % v/v total saturation). The resulting mixture was shaken again (500 rpm, 4^{o}C, 30 min.), after which the precipitated protein was centrifuged off with a Sigma 1-13 centrifuge (13000 rpm, 5 min., RT). The supernatant was decanted and another 650 µL of the saturated ammoniumsulfate was added to the supernatant (67 % v/v total saturation) after

which the mixture was again shaken gently (500 rpm, 30 mins, 4 ^{o}C). After 30 minutes 1 mL of this mixture was transferred to another Eppendorf tube and 12.5-200 µL of 25 wt% glutaraldehyde were added. After shaking (500 rpm, 4^{o}C, 3 hours) the suspended CLEA was centrifuged (13000 rpm, 10 min.) and washed 3 times with 1 mL buffer to remove all remaining glutaraldehyde and non-cross-linked protein. The CLEA was stored on ice in buffer until further use. To 500 µL of each of the three washing supernatants 500 µL of buffer were added. The resulting mixture was assayed for activity using hexanenitrile to determine enzyme leakage.

CLEA preparation using dextranpolyaldehyde

The oxidation of 6.6 g of dextran (from *Leuconostoc mesenteroides,* average M_w: 100 to 200 kDa) was carried out in 200 mL of water with 15.4 g of sodium metaperiodate (room temperature, 90 min). After stirring, the solution was dialysed with cellulose dialysis tubing (M_w cutoff: 12.4 kDa) against running tap water. The final volume of the dextranpolyaldehyde solution obtained is 225 mL.

Dextranpolyaldehyde CLEAs were prepared by adding different amounts of the dextranpolyaldehyde solution (125, 250, and 500 µL) to 250 µL of the supernatant of a 41 % v/v ammoniumsulfate cut. After the addition of the dextranpolyaldehyde, ammoniumsulfate is added to 70 % v/v total saturation. After shaking (500 rpm, 2 hours, 4 ^{o}C) the formed CLEA was resuspended in a $NaBH_4$ solution (1 mg/mL, 45 min., 4 ^{o}C). The reduced CLEA was centrifuged (13000 rpm, 10 min.) and washed 3 times with 1 mL buffer. The CLEA was stored on ice in buffer until further use.

HPLC analysis

Hexanenitrile, hexanamide and hexanoic acid concentration in the reaction were analysed by HPLC using a 4.6 x 50 Merck Chromolith SpeedROD RP-18e, eluent H_2O-ACN, 90:10 (v/v) containing acetic acid (0.1 %, v/v) at 1 mL/min and a column temperature of 21 ^{o}C. Hexanoic amide and hexanoic acid were detected using a Shimadzu SPD-10A VP UV-VIS detector at a wavelength of 210 nm. Hexanenitrile was detected using a Shimadzu RID 10A refractive index detector.

Acrylonitrile, acrylamide and acrylic acid concentration in the reaction were analysed by HPLC using a 4.6 x 50 Merck Chromolith SpeedROD RP-18e, eluent H_2O-ACN, 99:1 (v/v) containing acetic acid (0.1 %, v/v) at 1 mL/min and a column temperature of 21 ^oC. Acrylamide and acrylic acid were detected using a Shimadzu SPD-10A VP UV-VIS detector at a wavelength of 230 nm. Acrylonitrile was detected using a Shimadzu RID 10A refractive index detector.

Activity assay

Cell suspension and cell-free extract. To 1500 µL buffer 10 µL of cell-suspension or cell-free extract with a known protein concentration were added together with 10 µL of pure hexanenitrile (~54 mmol/L) in an Eppendorf tube. The tubes were shaken (700 rpm, 21^oC) for 5 minutes. After 5 minutes a 200 µL sample was withdrawn and mixed with the same amount of 1 M HCl to quench the reaction. The sample was centrifuged (13000 rpm, RT, 15 minutes) and the supernatant was injected directly on HPLC.

CLEA. To a certain amount (mg) of CLEA in an Eppendorf tube, 1000 µL of buffer and 15 µL of hexanenitrile were added (~120 mmol/L). The reaction was allowed to proceed for 5 minutes after which the CLEA was centrifuged off in 10-20 seconds. A 200 µL sample was withdrawn and mixed with the same amount of 1 M HCl to make sure that any accidentally transferred CLEA was deactivated. After centrifugation the supernatant was directly injected on HPLC.

Activity: one unit (U) of NHase will form one µmol of hexanoic amide per min.

Storage stability

Cell suspension, cell-free extract and CLEA were stored at 21^oC in buffer in the following concentrations: cell suspension (24 mg/mL protein), cell-free extract (16 mg/mL protein) and CLEA (3.7 mg/mL protein). The residual activities (%) were determined by following the activities of the different formulations during time using the activity assay and comparing these activities to the initial activities.

Recycling stability

Recycling stability was determined by carrying out a standard CLEA activity test. After the reaction the CLEA was washed three times using buffer to make sure all hexanoic amide was removed. After washing another activity assay was carried out and residual activity was determined.

Temperature stability

In 1000 µL of buffer, 20 µL of cells or cell-free extract and around 3.7 mg of CLEA were incubated at 20-50 °C for 20 minutes. After incubation the solution was cooled until it reached 21°C and residual activity of the different NHase formulations were determined in a standard activity test.

pH Profile

In 1000 µL of buffer, 20 µL of cells or cell-free extract and around 3.7 mg of CLEA were incubated at different pH values (pH 4-6: 0.01 M citrate buffer, pH 7-9: 0.01 M Tris-HCl buffer, pH 10-12: 0.01 M phosphate buffer) at 21°C for 20 minutes. After incubation activity was determined.

Starting concentration experiment

Whole cells, cell-free extract or CLEA of different amounts (necessary to assure the use of a total of 18 units in each reaction) were mixed with buffer to a volume of 1000 µL, after which 30 – 150 µl of acrylonitrile (2.9-13 %, v/v, 440-1980 mmol/L) were added. This solution was shaken at 21°C at 700 rpm for cell-free extract and whole cells and 900 rpm for the CLEA. Every 30 minutes, a 10 µL sample was withdrawn. The withdrawn sample was quenched with 1 M HCl. After centrifugation and, when necessary, dilution with Milli-Q water the sample was analyzed by HPLC. The reaction was monitored in this fashion until increase in amide concentration was no longer detected. In the case of CLEA, the catalyst was washed several times with buffer after the reaction was completed to remove all acrylamide. Subsequently residual activity of the catalyst was determined using the activity test.

Fed-batch experiment

Whole cells, cell-free extract or CLEA of different amounts (necessary to assure the use of a total of 18 units in each reaction) were mixed with buffer to a volume of 1000 µL, after which 20 µL of acrylonitrile (~300 mmol/L) were added. This solution was shaken at 21°C at 700 rpm for cell-free extract and whole cells and 900 rpm for the CLEA. Every 30 minutes, a 10 µL sample was withdrawn and 20 µL of acrylonitrile was added. The withdrawn sample was quenched with 1 M HCl. After centrifugation and, when necessary, dilution with Milli-Q water, the sample was analyzed by HPLC. The reaction was monitored in this fashion until increase in amide concentration was no longer detected. In case of the CLEA, the reaction was continued to a concentration of 2-3 mol/L of acrylamide, after which the CLEA was washed with buffer until acrylamide was no longer detected in the washing supernatant. After washing the CLEA residual activity was determined using the activity assay. The CLEA was stored on ice until the next fed-batch cycle was started.

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6

Synthesis of Aliphatic *(S)*-α-Hydroxycarboxylic Amides using a One-Pot Bienzymatic Cascade of Immobilised Oxynitrilase and Nitrile Hydratase

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Abstract

A one-pot bienzymatic cascade combining a hydroxynitrile lyase (*Manihot esculenta*, E.C. 4.1.2.10) and a nitrile hydratase (*Nitriliruptor alkaliphilus*, E.C. 4.2.1.84) for the synthesis of enantiopure aliphatic α-hydroxycarboxylic amides from aldehydes is described. Both enzymes were immobilised as cross-linked enzyme aggregates (CLEAs). Stability tests show that the nitrile hydratase CLEAs are sensitive to water-immiscible organic solvents as well as to aldehydes and hydrogen cyanide (HCN), but are remarkably stable and show useful activity in acidic aqueous environments of pH 4 - 5. The cascade reactions are consequently carried out by using a portionwise feed of HCN and moderate concentrations of aldehyde in acidic aqueous buffer in order to suppress the uncatalysed hydrocyanation background reaction. After optimisation, this method was used to synthesise five different kinds of aliphatic α-hydroxycarboxylic amides from the corresponding aldehydes with good yields and with enantiomeric purities comparable to those obtained for the α-hydroxynitriles in the microaqueous hydrocyanation using hydroxynitrile lyase and an excess of HCN.

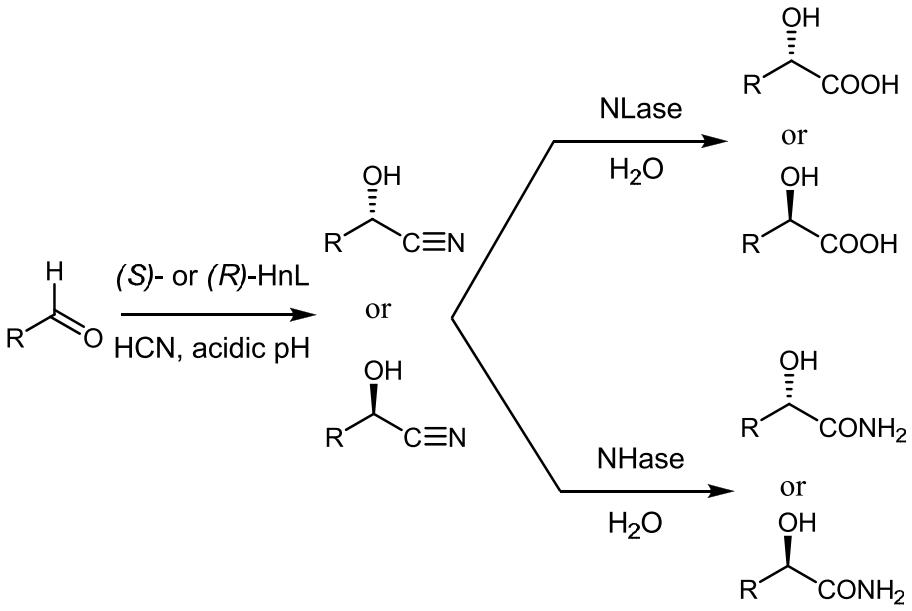
Introduction

Chiral α-hydroxycarboxylic amides, like the corresponding acids, are versatile building blocks in organic synthesis because of the ease of transformation of the functional groups in these molecules. In the last two decades, research was mainly focused on the development of biocatalytic synthesis routes to enantiopure α-hydroxycarboxylic acids.[1] However, a recent brainstorm session and cross company debate of the Pharmaceutical Roundtable identified a definite need for greener and more atom efficient methods of amide formation in the pharmaceutical industry.[2]

Enantiomerically pure *(R)*- and *(S)*-α-hydroxynitriles can be synthesised enzymatically from the corresponding aldehydes using an oxynitrilase (hydroxynitrile lyase, HnL, E.C. 4.1.2.10).[3] Chemical hydration or hydrolysis of these enantiopure nitriles using a strong acid will then give rise to the corresponding enantiopure carboxylic amides or acids. However, chemical hydration or hydrolysis is usually not compatible with sensitive functional groups and generates large amounts of salt waste. A greener route to α-hydroxycarboxylic acids comprises the asymmetric enzymatic hydrolysis of the cyano group using a stereoselective nitrilase (NLase, E.C. 3.5.5.1).[4] The remaining undesired cyanohydrin enantiomer can easily be racemised *in situ* under basic reaction conditions, resulting in a dynamic kinetic resolution (DKR) of the nitrile. The main limitation of this method is that there are only a few nitrilases available that are truly enantioselective and that this enantioselectivity is usually limited to mandelonitrile and its derivatives.

Besides the aforementioned chemoenzymatic routes, a novel method employing a bienzymatic one-pot cascade for the synthesis of *(S)*-α-hydroxycarboxylic acids was recently published. In this method an *(S)*-selective HnL from *Manihot esculenta* is combined with a non-selective NLase from *P. fluorescens* in a combi-CLEA or in the acidotolerant fungal host strain *Pichia pastoris*.[5] This fully enzymatic route to *(S)*-α-hydroxy acids is environmentally benign and compatible with a wide range of hydrolytically sensitive groups because of the mild reaction conditions.

An analogous bienzymatic cascade can be envisaged for the one-pot synthesis of α-hydroxycarboxylic amides by using a nitrile hydratase (NHase, E.C. 4.2.1.84) instead of a NLase in combination with a *(R)*- or *(S)*-HnL. Like NLases, NHases are industrially relevant enzymes.[6] Their main drawbacks are low stability in cell-free preparations and low to nonexistent enantioselectivity on most substrates tested, which makes the use of a DKR on α-hydroxynitriles practically impossible. Recently, a new NHase containing organism from soda lakes was isolated by our group[7] and subsequently the cell-free NHase from this organism was stabilised by the formation of CLEAs.[8] When a bienzymatic cascade of HnL and NHase is used for the production of α-hydroxycarboxylic amides, the low selectivity of the NHase is no longer a drawback since either an *(R)*- or *(S)*-selective HnL will impart its enantioselectivity on the reaction (Scheme 1).



Scheme 1 Access to *(R)*- and *(S)*-α-hydroxycarboxylic amides and acids using a one-pot bienzymatic cascade of HnL and NLase or HnL and NHase. The stereogenic center is set in the molecule by an *(R)*- or *(S)*-selective HnL. Subsequent hydration/hydrolysis is catalysed by an aselective NHase/NLase.

We initially studied the stability of NHase CLEAs under the conditions used for HnL catalysed hydrocyanation. After establishing the stability, the conditions of the

one-pot enzymatic cascade were optimised and consequently α-hydroxycarboxylic amides were produced from several different aliphatic aldehydes.

Results and Discussion

Stability of NHase CLEAs under HnL Hydrocyanation Conditions

HnL catalysed hydrocyanations are preferably carried out under acidic conditions (pH < 5) to suppress the competing unselective uncatalysed hydrocyanation,[9] which will cause erosion of the enantiomeric excess (*ee*) of the α-hydroxynitrile product. Biphasic aqueous-organic as well as micro-aqueous systems can help to further suppress the uncatalysed hydrocyanation.[10] The *Me*HnL CLEA can tolerate these conditions quite well, especially when diisopropylether (DIPE) is used as the water-immiscible organic solvent.[11]

For NHases, in contrast, this environment is far from ideal. In order to find out whether NHase CLEAs can still function under HnL hydrocyanation conditions, an assessment was made of their stability in DIPE/buffer mixtures of different composition. *Me*HnL CLEAs tolerate lyophilisation very well and can form a homogeneous suspension in DIPE, which can be kept for weeks in the refrigerator without loss of activity. In contrast, NHase CLEAs suffer from a high loss of activity during freeze drying and are most stable when stored in an aqueous environment.[8] Incubation of the NHase CLEAs in different DIPE/buffer mixtures resulted in an average activity loss of 40% in one hour. In addition to the low stability, it is cumbersome to suspend the NHase CLEAs in these mixtures, since the particles coagulate easily and tend to stick to the walls of the reaction vessel.

Since the use of biphasic as well as micro-aqueous systems was not feasible, the stability of the NHase CLEAs in low pH aqueous buffer systems was determined. Although the optimum pH of the NHase from *Nitriliruptor alkaliphilus* in CLEA formulation is located around 8 (Figure 6.1), it proved to be surprisingly stable at pH values 2-4 units below the optimum (Figure 6.2) and still had a useful activity under these conditions.

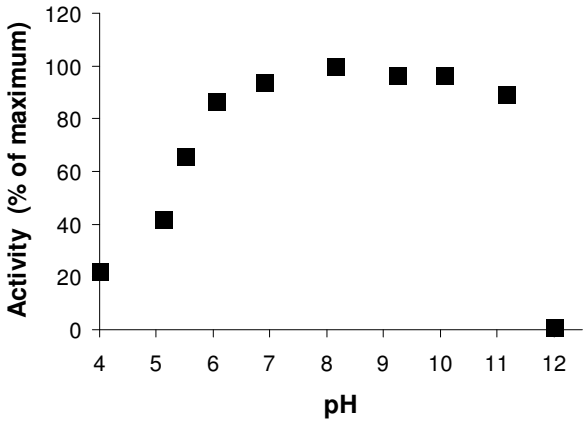


Figure 6.1 NHase CLEA pH activity profile.[8]

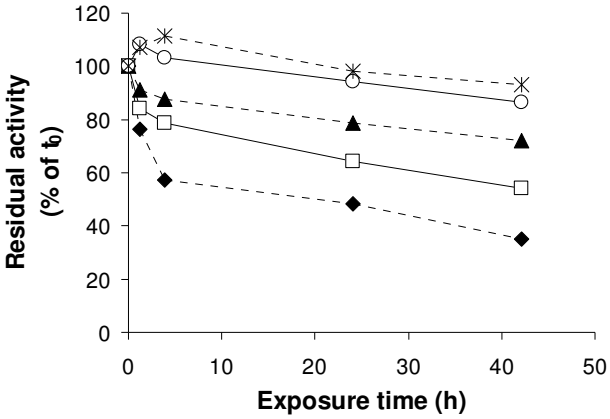


Figure 6.2 Deactivation of the NHase CLEAs caused by incubation in acidic buffer solutions at 21 °C (pH 4 – 5.5, 0.01 M citrate buffer; pH 6, MilliQ), pH 4 (♦), pH 4.5 (□), pH 5 (▲), pH 5.5 (○), pH 6 (*).

It is well known that NHases are highly sensitive to low to moderate cyanide concentrations.[12] Presumably, the deactivation and/or inhibition of the NHase by cyanide is caused by the strong interaction of cyanide with the cobalt or iron metal centre of this enzyme, which is assumed to be essential for catalysis.[13] NHases that can tolerate moderate to high cyanide concentrations may have interesting applications in the conversion of nitrile compounds that readily release cyanide because of decomposition, like α-hydroxy or α-aminonitriles.[14] In HnL catalysed hydrocyanation reactions, HCN is usually added in excess to drive the equilibrium to complete conversion. The stability of the NHase CLEA activity during incubation in the presence of different HCN concentrations is therefore of importance and was investigated (Figure 6.3). The activity was reasonably preserved during incubation in the presence of HCN concentrations ≤ 11 mM. HCN concentrations ≥ 21 mM resulted in severe loss of activity after a 30 min incubation. These results compare favourably with previously published results.[12] The activity of the CLEAs remained the same after washing them several times with buffer and storage for a week in an open atmosphere, indicating that the loss of activity is permanent. Nonetheless, the relatively high HCN tolerance of the NHase from *Nitriliruptor alkaliphilus* made this enzyme a good candidate for use in the cascade reaction system.

In general, aldehydes have the characteristic to bind to enzymes mostly through non-conjugated Schiff's base formation with the ε-amino groups of free lysine residues but they can also react with thiols, phenols and imidazoles.[15] These interactions with the enzyme can lead to a change in enzyme conformation and subsequent deactivation. In order to investigate the effects of aldehydes on the NHase CLEAs, these were incubated in the presence of different concentrations of acrolein, which was one of the intended substrates of the cascade reaction (Figure 6.4).

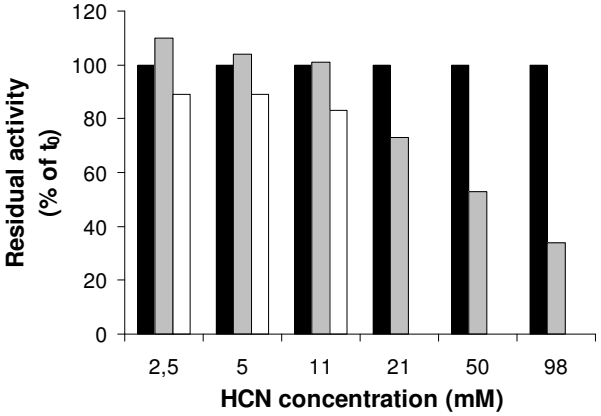


Figure 6.3 Deactivation of the NHase CLEAs caused by incubation in the presence of different concentrations of HCN at 21°C and pH 5.5, incubation of 0 min. (■), 30 min. (■), 90 min. (□).

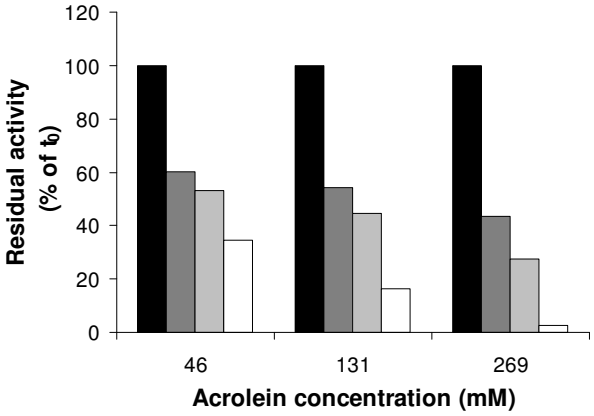


Figure 6.4 Deactivation of NHase CLEAs caused by incubation in presence of different concentrations of acrolein at 21 °C and pH 5.5, incubation of 0 h. (■), 2 h. (■), 3.5 h. (■), 18.5 h. (□).

Deactivation of the NHase CLEAs in the presence of moderate acrolein concentrations was quite severe. When the concentration of acrolein was monitored during experiments without HCN it is clear that the presence of NHase CLEAs is connected to a disappearance of acrolein (Figure 6.5). No alien peaks were detected during HPLC measurements, which might indicate that a part of the acrolein binds to the cross-linked enzyme aggregates. Apparently the acrolein is able to react either with amine groups that were not occupied during cross-linking of the NHase aggregates or with other essential functional groups. In contrast, propionaldehyde did not disappear during incubation with the NHase CLEAs, which indicates that the enzyme possibly reacts with the C=C double bond of acrolein in a Michael-type

addition. Nevertheless, the enzyme was deactivated by moderate concentrations of propionaldehyde.

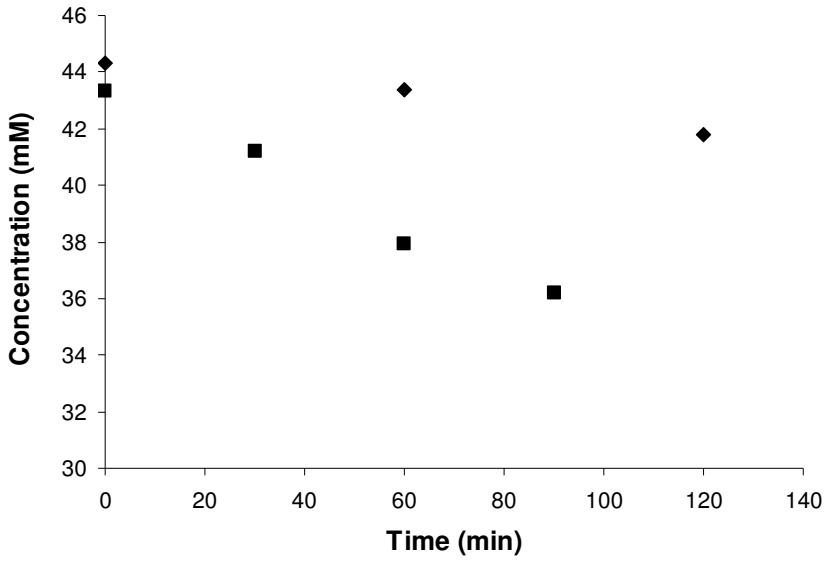


Figure 6.5 Disappearance of acrolein during incubation in buffer (0.01 M citrate buffer, pH 5.5) with and without the presence of NHase CLEAs, acrolein in buffer (♦), acrolein in buffer + 3.7 mg NHase CLEA (■).

During the preparation of NHase CLEAs with glutaraldehyde no reduction step was carried out since no leakage of free enzyme from the CLEA matrix was detected. However, non-conjugated Schiff's bases are considered to be unstable under acidic conditions and in theory are in equilibrium with the free amine and aldehyde. This equilibrium could be negatively affected by organic solvent, high temperature, low pH and the presence of other aldehyde species, leading to the rupture of the cross-linking bonds and subsequent deactivation. In order to fix the putative Schiff's bases formed by the ε-amino group of free lysines and glutaraldehyde, a reduction was carried out using sodium borohydride ($NaBH_4$). The effect of the reduction on the NHase CLEAs was noticeable by a change of colour from light brown to white. Reduction of the CLEAs using a $NaBH_4$ solution of 1 mg mL^{-1} resulted in an activity loss of 21 %, while > 90 % of the activity was lost when this $NaBH_4$ solution was refreshed 2 times during the 45 minute reduction process. This loss of activity might be the result of the reduction of the cysteinesulfinic and cysteinesulfenic acid ligands

by the reducing agent. Besides a slightly increased stability of the reduced CLEAs during incubation at pH 4, no noticeable effect of the reduction on CLEA stability in organic solvent or in the presence of acrolein was observed. The Schiff's bases formed by glutaraldehyde with the free amino groups of the enzyme are apparently very stable or cross-linking of the NHase CLEAs also takes place through the formation of other (stable) connections.[15]

In view of the results discussed above, carrying out a one-pot bienzymatic cascade using HnL and NHase to synthesise α-hydroxyamides from aldehydes and HCN is affected by several severe limitations. Because of the bad performance of the NHase CLEAs in DIPE mixtures, the reactions have to be carried out in aqueous environment and, hence, the uncatalysed hydrocyanation has to be suppressed by lowering the pH of the reaction as much as possible. Because of the low HCN tolerance of the metalloenzyme, the HCN concentration in the reaction should be kept low. A low HCN concentration will also have the benefit of reducing the uncatalysed hydrocyanation reaction rate and since the NHase will convert the formed α-hydroxynitrile to the stable amide, the equilibrium will shift to the right without the need of an HCN excess. The preferred mode of carrying out the cascade reactions was therefore the fed-batch addition of portions of HCN to modest aldehyde concentrations in acidic aqueous environment.

HnL-NHase One-Pot Bienzymatic Cascade

Because of the unfavourable reaction equilibrium and the low *ee*s obtained in the *Me*HnL catalysed hydrocyanation of acrolein,[11] this challenging aldehyde was chosen as the substrate for optimising the cascade reaction conditions, since changes in reaction conditions together with enzyme ratio were expected to have a more profound impact on the *ee* of the amide product.

As was previously mentioned, the uncatalysed background hydrocyanation reaction should be suppressed as much as possible, because it is unselective and will deteriorate the *ee* of the α-hydroxynitrile intermediate and consequently of the final amide product. In order to investigate the effect of the pH on the background reaction in more detail, the latter was monitored for acrolein under cascade reaction

conditions (5 mM HCN, 45 mM acrolein, 21 °C) at different pH values (Figure 6.6). As expected, lowering the pH of the cascade reaction decreased the rate of the uncatalysed hydrocyanation.

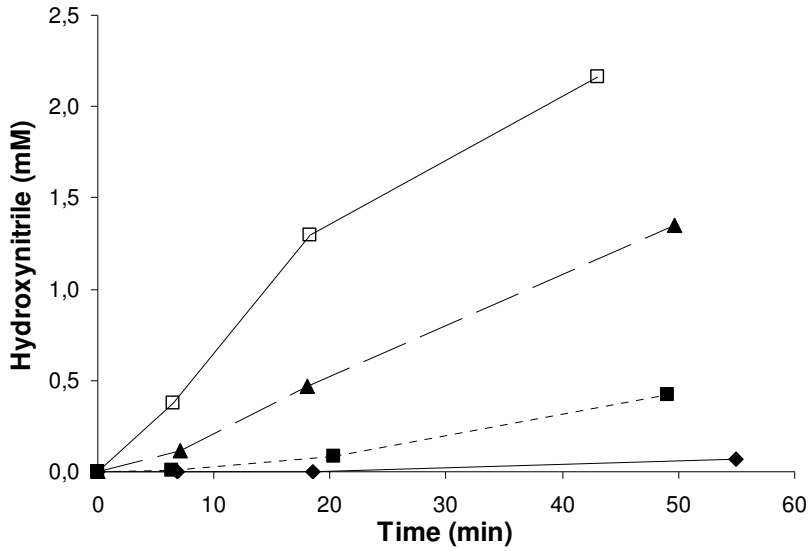
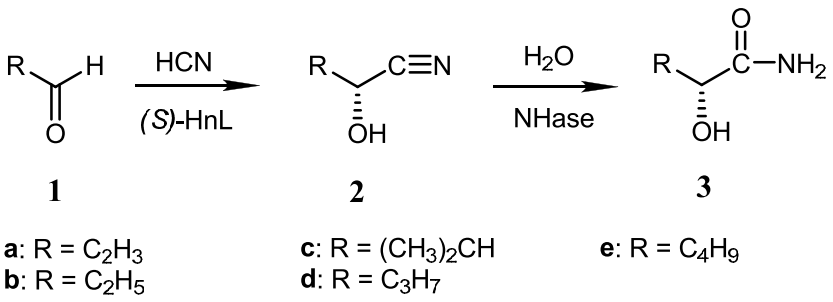


Figure 6.6 Rate of the uncatalysed hydrocyanation of acrolein at different pH under cascade reaction conditions, pH 4 (♦), pH 4.5 (■), pH 5 (▲), pH 5.5 (□).

The relative contribution of the background reaction is also dependent on the rate of enzymatic hydrocyanation. An increase of HnL activity in the cascade reaction would decrease the effect of the background reaction on the *ee* of the intermediate nitrile product. However, there is a limit to the amount of HnL that can be added to the cascade reaction, since its cascade partner should still be able to keep the intermediate nitrile concentration low to prevent chemical and/or enzymatic racemisation. The activity ratio of the two enzymes in this cascade is therefore an important variable.

During the cascade reaction studies (Scheme 6.2), the HnL activity added to the reaction mixture was fixed and the NHase activity was varied. The activity of the HnL CLEA for acrolein under cascade reaction conditions at pH 4 was 0.98 μmol min^{-1} mg^{-1}. During the cascade reactions a fixed amount of 1.8-1.9 mg mL^{-1} of HnL CLEA was used, which enabled reasonably fast reactions without building up high

concentrations of intermediate nitrile. Under these conditions the relative background reaction during the cascade reaction of acrolein (**1a**) was 3.1 % or less (Table 6.1).



Scheme 6.2 Synthesis of aliphatic *(S)*-α-hydroxyamides using a bienzymatic one-pot cascade of the *(S)*-HnL from *Manihot esculenta* and the NHase from *Nitriliruptor alkaliphilus*.

Table 6.1 HnL-NHase one-pot bienzymatic cascade reactions with **1a** (45-46 mM) at different pH, 21 °C, and a maximum HCN concentration of 5 mM.

pH	Background (%)[a]	Factor[b]	**3a** produced (mM)	*ee* (%) *(S)*
5.5[c]	3.1	12	24.4	35
5.5[c]	3.1	24	26.6	35
5[c]	1.6	24	24.8	39
4.5[d]	0.5	24	20.0	43
4.5[e]	0.5	24	35.5	43
4[c]	0.1	24	28.1	39
4[e]	0.1	48	40.0	48

[a.] Relative background reaction when using 1.8-1.9 mg HnL CLEA (T = 21°C)

[b.] Ratio of NHase activity to HnL activity. NHase activity was measured for the hydration of **2a** at pH 6 and HnL activity was measured for the production of **2a** under cascade reaction conditions at pH 4.

[c.] Reaction was stopped after spiking 5 times with 5 mM HCN

[d.] Reaction was stopped after spiking 4 times with 5 mM HCN

[e.] Reaction was continued to the point were **1a** was completely converted.

The pH of the cascade reaction clearly had an effect on the final *ee* of **3a**. This is to be expected, since the competing unselective background hydrocyanation rate increases at a higher pH. The drop in *ee* between pH 4.5 and the first experiment at pH 4 is most probably the result of enzymatic racemisation of **2a**, since moderate concentrations of **2a** were detected by HPLC at this pH (Figure 6.7).[11] Apparently, it

was not possible for the NHase to keep up with the HnL in this situation. When the NHase activity at pH 4 was doubled, the nitrile intermediate was no longer detected and the enantiomeric purity of the amide improved. A twofold increase of the relative NHase activity in the cascade reaction at pH 5.5, in contrast to pH 4, did not lead to an improvement in product *ee*, indicating that the conversion of the α-hydroxynitrile intermediate by the NHase CLEA is fast enough to prevent enzymatic racemisation of **2a** at this pH.

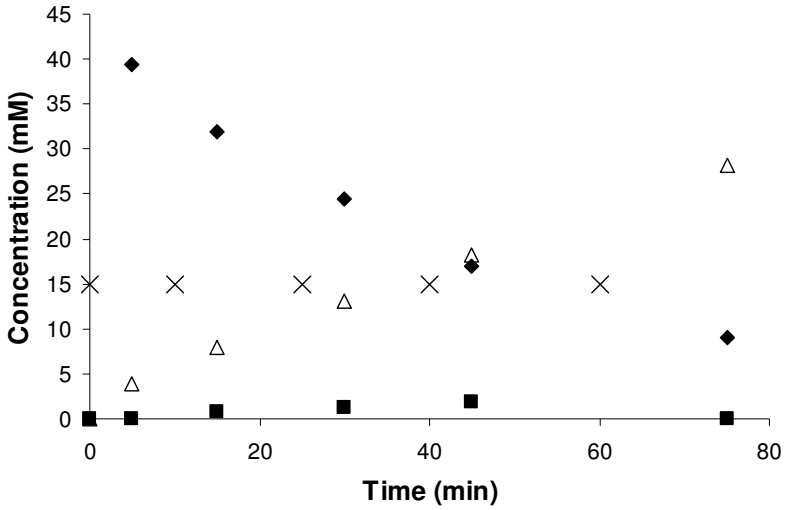


Figure 6.7 HnL-NHase one-pot bienzymatic cascade conversion of acrolein at pH 4, 1a (♦), 2a (■), 3a (Δ), HCN spike (x).

Complete conversion of **1a** at pH 4.5 did not result in a lower *ee* of **3a** compared to the reaction where **1a** was not fully converted. This indicates that stopping the reaction prematurely to maintain a favourable equilibrium and to prevent enzymatic racemisation was not necessary. The yield on **1a** was between 77 and 89 %, depending on the conditions and time course of the reaction (Figure 6.7). The loss of **1a** in the reaction is probably caused by attachment of the substrate to the enzyme, as was demonstrated before (Figure 6.5). In these reactions, the formation of α-hydroxy-3-butenoic acid was not observed by HPLC, GC, and NMR.

Although the HnL-NHase cascade can be used for conversion of **1a** into **3a**, the ee of the final amide product is poor. However, the *Manihot esculenta* HnL is known

to have a rather low selectivity in converting acrolein (47-56 %).[11,16] The fact that the HnL catalysed hydrocyanation reactions are usually carried out in organic solvent also has to be taken into account, since the cascade reaction is carried out in aqueous buffer only. In order to attempt the production of α-hydroxycarboxylic amides with higher enantiomeric purity, the same cascade reaction was used for the conversion of aliphatic aldehydes for which the HnL from *Manihot esculenta* is known to have a higher selectivity (Table 6.2).

Table 6.2 HnL-NHase one-pot bienzymatic cascade reactions of **1b – 1e** (35-45 mM) at pH 4.5, 21 °C, and a maximum HCN concentration of 5 mM. Unless stated otherwise, reactions were stopped after full conversion of the aldehyde.

Compound	**3** produced (mM)	Factor[a]	*Ee* (%) *(S)*	*ee* (%) *(S)*-**2**[16,17]
b	44	24	86	91
c[b]	28	24	84	95
c	35	48	88	95
d	41	24	88	88
e	43	48	90	91

[a.] Ratio of NHase activity to HnL activity. NHase activity is measured for the hydration of **2a** at pH 6 and HnL activity is measured for the production of **2a** under cascade reaction conditions at pH 4.

[b.] Full conversion was not achieved with this amount of NHase.

The *ee* of the amide products significantly improved when aldehydes were used for which the *Me*HnL is known to show a higher selectivity. In all cases, except for **d** and **e**, the *ee* of the amide products is lower than the *ee* of the nitriles as reported in literature for *Me*HnL catalysed hydrocyanations in microaqueous systems using an excess of HCN. The higher *ee*'s obtained for compounds **3d** and **3e** could be the result of a low preference for the corresponding *(S)*-α-hydroxy nitriles by the NHase.

The yields of the α-hydroxy amide on the aldehyde are > 95%, which contrasts with the lower yields obtained when acrolein is used as the substrate. Unlike acrolein, the aliphatic aldehydes did not disappear during the reaction because of possible attachment to the enzyme. No traces of the corresponding α-hydroxycarboxylic acids were detected on HPLC and GC during these experiments.

Conclusions

A bienzymatic one-pot cascade reaction to synthesise aliphatic α-hydroxycarboxylic amides using *Me*HnL and NHase CLEAs was made possible by using aqueous acidic citrate buffer of pH 4 – pH 5.5 as reaction medium containing moderate concentrations of aldehyde and the subsequent portionwise feed of HCN. After optimisation of the pH together with the HnL – NHase activity ratio in the reaction, five aliphatic aldehydes were successfully converted into the α-hydroxycarboxylic amides. Except for acrolein, the aldehydes were converted into the stable α-hydroxycarboxylic amides with good yield and high *ee* without the need for organic solvent and an excess of HCN.

The *ee* of the α-hydroxycarboxylic amides as well as the stability of the immobilised nitrile hydrating biocatalyst can probably be further improved by the controlled continuous addition of low concentrations of aldehyde and pure HCN and by co-immobilisation of the two biocatalysts in the form of a combi-CLEA.

An NHase with an inherent or designed increased stability in microaqueous systems could further increase the performance of this system and the substrate scope of the cascade reaction system could possibly be broadened to aromatic aldehydes by using an NHase with a higher activity for aromatic nitriles.

Experimental

General comments

Reactions and stability tests were carried out in Eppendorf tubes using a ThermoTWISTER comfort shaker of QUANTIFOIL Instruments. Freeze drying was performed by a Christ Alpha 2-4 freeze dryer at a reduced pressure of 0.1 mbar and a temperature of -80 ^{o}C, after freezing the samples with liquid N_2. An Eppendorf 5415R centrifuge was used for centrifugation at 13200 rpm. The temperature of the centrifuge was adjusted according to the needs of the experiment.

Materials

Semi-purified *(S)*-hydroxynitrile lyase from *Manihot esculenta* was obtained from Jülich Fine Chemicals (now Codexis). The NHase was produced by cultivation and induction of the haloalkaliphilic actinobacterium *Nitriliruptor alkaliphilus*.[7] NHase and *Me*HnL CLEAs were prepared as described previously.[8,11] NHase CLEAs were stored at + 4 °C in Tris-HCl buffer (0.01 M, pH 8), while the *Me*HnL CLEAs were stored at - 20 °C in lyophilised form. Hexanenitrile (98 %, Aldrich), hexanamide (98 %, Aldrich), sodium borohydride (> 96 %, Fluka), diisopropyl ether (DIPE, > 99 %, Fluka), acrolein (> 95 %, Fluka), propionaldehyde (99+ %, Janssen), isobutyraldehyde (99+ %, Acros), butyraldehyde (> 99.5 %, Aldrich), valeraldehyde (> 97 %, Fluka), α-hydroxybutyronitrile (Fluka, purum), α-hydroxybutyric acid (Fluka, > 97 %), and α-hydroxycaproic acid (Aldrich, 98 %) were used in the experiments as received without additional purification. A 2 M solution of hydrogen cyanide in DIPE was prepared from sodium cyanide (98+ %, Acros) as described previously.[18] **Warning:** both sodium cyanide and HCN are highly poisonous. They should always be handled in a fume cupboard with a good draught. It is strongly advised to use a well-calibrated HCN detector during HCN work.

Analytical Procedures

HPLC analysis: The progress of the reactions was monitored by HPLC using one or three 4.6 x 50 Merck Chromolith SpeedROD RP-18e columns, depending on the polarity of the compounds analysed. The eluent (MilliQ with 0.1 v % TFA) was pumped through the column(s) at a flowrate of 1 mL min^{-1} using a Waters 590 HPLC pump. The HPLC analyses were carried out at room temperature. All compounds were detected using a Shimadzu RID 10A refractive index detector and/or a Shimadzu SPD-10A VP UV-VIS detector at a wavelength of 210 nm (Table 6.3).

Table 6.3 Retention times on HPLC of the different compounds in the cascade reaction.

		Retention time [min]		
Compound	Column #	**1**	**2**	**3**
a	3	5.2	4.4	3.2
b	3	4.8	4.6	3.5
c	3	9.4	12.2	5.3
d	3	8.9	10.9	5.0
e	1	7.6	11.6	4.0

GC analysis: Enantiomeric excess (*ee*) of the formed aliphatic α-hydroxycarboxylic amides **3a – d** was determined using a 25 m Chirasil dex CB column with i.d. 0.32 mm and d_f 0.25 µm on a Shimadzu GC-17A gas chromatograph equipped with a FID detector ($T_{injector}$ = 200 °C, $T_{detector}$ = 200 °C). The carrier gas used was N_2 at a column flowrate of 5.92 mL min^{-1}. The *(S)*- and *(R)*-enantiomers were separated using the temperature programs in Table 6.4. The enantiomers of compound **3e** were separated using a 50 m Chiradex GTA column with i.d. 0.25 mm and d_f 0.12 µm on a Shimadzu GC-2010 gas chromatograph equipped with a FID detector ($T_{injector}$ = 200 °C, $T_{detector}$ = 200 °C). The carrier gas used was N_2 at a column flowrate of 5.00 mL min^{-1}.

Table 6.4 Retention times and temperature programs used for separation of the *(S)*- and *(R)*-enantiomers of the formed aliphatic α-hydroxycarboxylic amides.

Compound	T program (°C)	R_t *(R)* (min)[a]	R_t *(S)* (min)[a]
3a	100	34	36
3b	100	37	39
3c	105	43	46
3d	105	21[b]	25[b]
3e	115	39[c]	36[c]

[a.] Since the *Me*HnL is *(S)*-selective, the peak with the largest area was designated as the *(S)*-enantiomer

[b.] This α-hydroxycarboxylic amide was first derivatised by a one hour incubation in a mixture of acetic anhydride (0.8 mL) and pyridine (0.8 mL) in dichloromethane (10 mL).

[c.] The elution of the *(S)*- and *(R)*- enantiomers on this column are in reversed order.

Cyanide concentration: Cyanide concentrations in the reactions and stability tests were determined by using the Merck Spectroquant® cyanide test kit for the determination of free and readily liberated cyanide in water according to the manual of the manufacturer. Cyanide stock solutions for calibration were prepared by dissolving potassium cyanide in a KOH solution (35 mM).

Standard Activity Assay

NHase CLEAs were suspended in buffer (1000 µL, 0.01 M Tris-HCl of pH 8, unless stated otherwise) in an Eppendorf tube and subsequently hexanenitrile was added (15 µL, ~120 mM). The reaction was allowed to proceed for 5 minutes after which the CLEAs were centrifuged off in 10-20 seconds. A sample (200 µL) was withdrawn from the supernatant and mixed with the same amount of HCl (1 M) to make sure that any accidentally transferred CLEA was deactivated. After centrifugation the supernatant was directly injected on HPLC.

NHase CLEA reduction

Batches of NHase CLEA (3.7 mg) were reduced using two different methods. Method A: the addition of $NaBH_4$ (1000 µL, 1 mg mL^{-1}) and subsequent shaking for 45 minutes at 4 ^{o}C. Method B: the addition of $NaBH_4$ (1000 µL, 1 mg mL^{-1}) and subsequent shaking for 45 minutes at 4 ^{o}C, where after 15 and 30 minutes the suspension was centrifuged and fresh $NaBH_4$ (1000 µL, 1 mg mL^{-1}) was added. After the reduction step, the CLEAs were washed three times with Tris-HCl buffer (0.01 M, pH 8) and the residual activity was determined using the standard activity assay.

NHase CLEA Stability Tests

Two-phase DIPE-buffer system: NHase CLEAs were incubated in mixtures of DIPE (50 v %, 90 v % and > 99 v %) and Tris-HCl buffer (0.01 M, pH 8). After an incubation time of one hour (900 rpm, 21 ^{o}C), the CLEAs were washed 3 times with Tris-HCl buffer (0.01 M, pH 8) to remove all traces of DIPE. The residual activities were determined by the standard activity assay.

Acidic pH: NHase CLEAs were incubated in buffers with different acidic pH values (pH 4-5.5: 0.01 M citrate buffer) and in MilliQ (pH ~ 6) at 21 °C. The residual activities after different incubation times were determined by the standard activity assay. Changing buffers for incubation was carried out by washing the CLEAs 3 times with the corresponding buffer.

HCN: NHase CLEAs were washed 3 times with citrate buffer (0.01 M, pH 5.5). To a mixture of this buffer (1000 µL) and the CLEAs, HCN was added (2.5, 5, 11, 21, 50, and 98 mM). These mixtures were then shaken at 900 rpm and 21 °C. After 30 and/or 90 minutes of incubation the incubation mixture was centrifuged and the CLEAs washed 3 times with citrate buffer. The residual activity was determined by the standard activity test at pH 5.5. After the activity test the CLEAs were washed 3 times with Tris-HCl buffer (0.01 M, pH 8) and stored at + 4 °C in an open atmosphere. Activity of the CLEAs was reassessed after one week of storage.

Acrolein and propionaldehyde: NHase CLEAs were washed 3 times with citrate buffer (0.01 M, pH 5.5). To a mixture of this buffer (1000 µL) and the CLEAs, acrolein or propionaldehyde were added (46, 131, and 269 mM). After 2, 3.5 and 18.5 hours of incubation (900 rpm, 21 °C), the incubation mixture was centrifuged and the CLEAs washed 3 times with citrate buffer. The residual activities were determined by the standard activity test at pH 5.5.

Preparation of the Racemic Amides

Racemic **2a** was prepared according to a literature procedure.[19] Racemic **2b** (purum) was purchased at Fluka. Racemic **3a** and **3b** were prepared by adding racemic **2a** and **2b** in fed-batch fashion to a suspension of NHase CLEAs in MilliQ (21 °C, 900 rpm). After the hydration reaction was finished the CLEA particles were spun down by centrifugation and the supernatant was removed. Freeze drying of the obtained supernatant resulted in fluffy white amide crystals of high purity. Racemic **2c – 2e** were produced by adding equimolar amounts of **1c-1e** and HCN to a suspension of HnL CLEAs in citrate buffer (pH 4.5, 0.01 M), after which the reaction was allowed to proceed overnight. Racemic **3c – 3e** were then prepared by the removal of the HnL CLEAs by centrifugation, after which NHase CLEAs were added

to the supernatant of the enzymatic hydrocyanation reaction. After the hydration reaction was completed, the NHase CLEAs were removed by centrifugation and the supernatant was subsequently freeze dried to obtain the racemic amide crystals.

HnL-NHase Cascade

In order to obtain better suspension behaviour, HnL CLEAs were incubated in citrate buffer (0.01 M) under reduced pressure to remove air from the very fluffy lyophilised particles before the reaction. To a suspension of NHase and HnL CLEAs with a certain activity ratio in citrate buffer (0.01 M, pH 4-6), aldehydes were added (30-50 mM). At these concentrations the aldehydes were completely soluble in citrate buffer. After sampling for determination of the exact aldehyde starting concentration, the cascade reaction was initiated by the addition of HCN (5 mM). The reaction was spiked with portions of HCN (5 mM) several times. The time intervals between the HCN spikes depended on the speed of hydration of the α-hydroxynitrile intermediate. Sampling was carried out by stopping the reaction with a 30 second centrifugation after which a sample (50 µL) was withdrawn. The sample was directly injected on the HPLC and the excess sample liquid was returned to the reaction. At the end of the reaction the NHase and HnL CLEAs were spun down by a 10 minute centrifugation and subsequently all supernatant was removed. After removing excess HCN under vacuum, the supernatant was freeze dried, after which white fluffy crystals were obtained. Citrate, which is an impurity in the crystalline product, can be removed by the addition of methanol in which the buffer does not dissolve. After removing insoluble citrate by filtration, the enantiomeric excess of the final product was determined by chiral GC as discussed previously.

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Summary

The enzymatic conversion of a nitrile into the corresponding acid is one of the pathways of nitrile metabolism in Nature. This reaction can be catalysed either by a nitrilase or by a nitrile hydratase/amidase coupled system. Nitrile hydratase (NHase, E.C. 4.2.1.84) catalyses the conversion of a nitrile into the corresponding amide and was first discovered 30 years ago in studies on the microbial degradation of toxic cyano-group-containing compounds by Asano and co-workers. Ever since their discovery, NHases have attracted an increasing attention as green catalysts for the synthesis of amides in industry as well as academia. The application of NHases in organic synthesis is the topic of this thesis.

Chapter 1 starts with a general introduction on sustainability, green chemistry and biocatalysis. An overview is then given of the different enzymes in the nitrile forming and degrading pathways in Nature. Furthermore, the characteristics of NHases as well as examples of their successful application in industry are discussed in detail.

In **Chapter 2**, an attempt was made to isolate new NHase containing organisms by studying the utilisation of isobutyronitrile, as a carbon and nitrogen source, under haloalkaline conditions by microbial communities from natural soda lake sediments and soda soils from Russia and Mongolia. These studies resulted in the selection and isolation of two new NHase containing organisms. The new NHase containing actinobacterium *Nitriliruptor alkaliphilus* was isolated from soda lake sediments and the new NHase containing *Bacillus alkalinitrilicus* was isolated from soda soils. Both of the new bacterial strains were moderately salt-tolerant alkaliphiles with a pH range for growth from pH 7.0 up to 10.5. However, both their NHase and amidase activities had a near neutral pH optimum, indicating an intracellular localisation of these enzymes.

The induction of NHase activity in *Nitriliruptor alkaliphilus* was examined in more detail in **Chapter 3**. Without the presence of a nitrile, carboxylic amide, or carboxylic

acid in the fermentation broth, very little or no NHase activity was detected. It was found that the inducing agent concentration during growth should be maintained at a high level for maximum activity. The whole cell nitrile hydrating biocatalyst was most active on C_4 and C_5 straight chain saturated aliphatic nitriles, but also easily accepted saturated and unsaturated branched aliphatic nitriles, as well as (hetero)aromatic and arylaliphatic nitriles. Although highly stable at low temperatures, the cells rapidly started losing activity at temperatures higher than 37 ^{o}C. The pH optimum of the free enzyme was 8, while the whole cells had a broad pH optimum from 7 - 10. The cell-free NHase was purified and the α- and β-subunits of the enzyme were found to have a molecular weight of around 26 and 28 kDa. The characteristic C**T**LCSC domain for Co-containing NHases was identified by a PCR based screening method using degenerate primers.

In **Chapter 4** the purified cell-free Co-containing NHases from *Nitriliruptor alkaliphilus* (iso2), *Rhodopseudomonas palustris* HaA2 (HaA2), *Rhodopseudomonas palustris* CGA009 (009), and *Sinorhizobium meliloti* 1021 (1021), as well as the Fe-containing NHase from *Rhodococcus erythropolis* AJ270 (AJ270) were probed for their enantioselectivity on a group of different chiral nitriles. It was found that the presence of an alkyl group on the α-position of 2-phenylacetonitrile was vital to induce a good enantioselectivity in the Co-containing NHases. Enantiomeric ratios of > 100 were found for the NHases from HaA2 and CGA009 on 2-phenylpropionitrile. In contrast, the Fe-containing NHase was practically aselective for all the different α-phenylacetonitriles.

In general, at least one bulky group in close proximity to the α-position of the chiral nitriles seemed to be necessary for enantioselectivity with all NHases tested. Substituents on the aromatic ring had a positive influence on the enantioselectivity of the AJ270 and iso2 NHases, but decreased the selectivity of the other enzymes. Enantiomeric ratios of 80 and >100 for AJ270 and iso2, respectively, were found for naproxennitrile and 3-(1-cyanoethyl)benzoic acid was hydrated to the corresponding amide by iso2 with an enantiomeric ratio of > 100.

In **Chapter 5**, the relatively unstable cell-free NHase from *Nitriliruptor alkaliphilus* was immobilised and stabilised in the form of a cross-linked enzyme aggregate (CLEA). CLEAs were prepared by using ammonium sulfate as an aggregation agent followed by cross-linking with glutaraldehyde. The effect of different glutaraldehyde concentrations on the recovery of enzyme activity in the CLEA and enzyme leakage from the CLEA matrix was investigated. Although activity recovery was low (21 %) the CLEA facilitated easy separation and recycling of the NHase. It was also found that the NHase CLEA had substantially increased storage stability as well as increased operational stability during exposure to high concentrations of acrylamide and acrylonitrile compared to that of the NHase in the crude cell-free extract and whole cell formulation.

Finally, the CLEA of the NHase from *Nitriliruptor alkaliphilus* was combined with the CLEA of a hydroxynitrile lyase from *Manihot esculenta* in a one-pot bienzymatic cascade for the synthesis of enantiopure aliphatic α-hydroxycarboxylic amides from aldehydes in **Chapter 6**. Stability tests showed that the NHase CLEAs were sensitive to water-immiscible organic solvents as well as to aldehydes and hydrogen cyanide (HCN), but were remarkably stable and showed useful activity in acidic aqueous environments of pH 4 - 5. The cascade reactions were consequently carried out by using a portionwise feed of HCN and moderate concentrations of aldehyde in acidic aqueous buffer in order to suppress the uncatalysed hydrocyanation background reaction. After optimisation, this method was used to synthesise five different kinds of aliphatic α-hydroxycarboxylic amides from the corresponding aldehydes with good yields and with enantiomeric purities comparable to those obtained for the α-hydroxynitriles in the microaqueous hydrocyanation using hydroxynitrile lyase and an excess of HCN.

Samenvatting

De enzymatische omzetting van een nitril naar het zuur is één van de reactiewegen die in de natuur gebruikt wordt voor het nitrilmetabolisme. Deze reactie kan gekatalyseerd worden door een nitrilase of door een gekoppeld systeem bestaande uit een nitrilhydratase en een amidase. Het enzym nitrilhydratase (NHase, E.C. 4.2.1.84) katalyseert de omzetting van een nitril naar het amide en werd aanvankelijk 30 jaar geleden ontdekt door Asano en medewerkers tijdens studies naar de microbiologische afbraak van giftige nitrillen. Sinds hun ontdekking is de belangstelling voor NHases als groene katalysatoren voor de synthese van amides in zowel de industriële als de academische wereld alleen maar toegenomen. De toepassing van NHases in de organische synthese is het onderwerp van dit proefschrift.

Hoofdstuk 1 vangt aan met een algemene introductie over duurzaamheid, groene chemie en biokatalyse. Vervolgens wordt er een overzicht gegeven van de verschillende enzymen die werkzaam zijn in de nitrilvormende en nitrilafbrekende reactiewegen in de natuur. Hierna worden de eigenschappen van NHases samen met voorbeelden van succesvolle toepassingen in de industrie in detail behandeld.

In **Hoofdstuk 2** werd een poging ondernomen om nieuwe NHasebevattende organismen te isoleren. Het gebruik van isobutyronitril als een koolstof- en stikstofbron onder haloalkalische condities door microbiële gemeenschappen uit natuurlijke zoutmeersedimenten en zoutgronden in Rusland en Mongolië werd onderzocht. Deze studies hadden de selectie en de isolatie van twee nieuwe NHasebevattende organismen tot resultaat. De nieuwe NHasebevattende actinobacterie *Nitriliruptor alkaliphilus* werd geïsoleerd uit zoutmeersedimenten en de nieuwe NHasebevattende *Bacillus alkalinitrilicus* werd geïsoleerd uit zoutgronden. De nieuwe stammen waren beiden gematigd zouttolerante alkalifielen met een pH gebied voor groei tussen de pH 7 en pH 10.5. Zowel hun NHase- als

amidaseactiviteiten hadden echter een pH optimum in de buurt van neutraal, hetgeen wijst op een intracellulaire lokalisatie van deze enzymen.

De inductie van NHaseactiviteit in *Nitriliruptor alkaliphilus* werd in meer detail onderzocht in **Hoofdstuk 3**. Zonder de aanwezigheid van een nitril, amide, of zuur in het fermentatiemedium werd heel weinig tot geen NHaseactiviteit gedetecteerd. Voor maximalisatie van de NHaseactiviteit was het van belang om de concentratie van de inducerende stof gedurende de fermentatie op een hoog niveau te houden. De activiteit van de hele cellen voor nitrilhydratatie was het hoogst met C_4 en C_5 verzadigde alifatische nitrillen met rechte ketens, maar verzadigde en onverzadigde alifatische nitrillen met vertakte ketens werden ook gemakkelijk omgezet, evenals (hetero)aromatische en arylalifatische nitrillen. Hoewel de activiteit van de cellen zeer stabiel bleek te zijn bij lage temperaturen, ging deze snel achteruit bij temperaturen hoger dan 37 ^{o}C. Het pH optimum van het vrije enzym was 8, terwijl de hele cellen een breed pH optimum hadden van 7 – 10. Het celvrije NHase werd in dit hoofdstuk gezuiverd en de grootte van de α- en de β-subunits van het enzym werd vastgesteld op 26 en 28 kDa. Het karakteristieke C**T**LCSC domein voor Co-bevattende NHases werd geïdentificeerd door gebruik te maken van een op PCR gebaseerde screeningsmethode met gedegenereerde primers.

In **Hoofdstuk 4** werden de gezuiverde celvrije extracten van de Co-bevattende NHases uit *Nitriliruptor alkaliphilus* (iso2), *Rhodopseudomonas palustris* HaA2 (HaA2), *Rhodopseudomonas palustris* CGA009 (009), en *Sinorhizobium meliloti* 1021 (1021) samen met het Fe-bevattende NHase uit *Rhodococcus erythropolis* AJ270 (AJ270) getest op hun enantioselectiviteit met een groep van verschillende chirale nitrillen. De aanwezigheid van een alkylgroep op de α-positie van 2-fenylacetonitril was van belang voor een goede enantioselectiviteit met de Co-bevattende NHases. Enantiomere ratios van > 100 werden gevonden voor de NHases uit HaA2 en CGA009 voor 2-fenylpropionitril. Het Fe-bevattende NHase was daarentegen bijna volledig aselectief voor al de verschillende α-fenylacetonitrillen.

In het algemeen was tenminste één grote groep in de directe omgeving van de α-positie in de chirale nitrillen noodzakelijk voor het induceren van enantioselectiviteit

bij alle NHases. Substituenten op de aromatische ring hadden een positieve invloed op de enantioselectiviteit van de AJ270 en iso2 NHases, maar hadden een negatieve invloed op de andere enzymen. Enantiomere ratios van respectievelijk 80 en >100 werden gevonden voor AJ270 en iso2 bij de omzetting van naproxennitril. 3-(1-Cyanoethyl)benzoëzuur werd gehydrateerd naar het overeenkomstige amide door iso2 met een enantiomere ratio van > 100.

In **Hoofdstuk 5** werd het relatief instabiele celvrije NHase uit *Nitriliruptor alkaliphilus* geïmmobiliseerd en gestabiliseerd in de vorm van een gecrosslinked enzymaggregaat (CLEA). De CLEA's werden gemaakt door ammoniumsulfaat te gebruiken als aggregatiemiddel gevolgd door crosslinking met glutaaraldehyde. Het effect van verschillende glutaaraldehyde concentraties op de herwinning van de enzym aktiviteit in de CLEA en op het lekken van enzym uit de CLEA matrix werd onderzocht. Hoewel de aktiviteitsherwinning laag was (21 %), zorgde de CLEA voor een makkelijke scheiding en hergebruik van het NHase. Er werd gevonden dat de NHase CLEA een substantieel hogere opslagstabiliteit had evenals een hogere operationele stabiliteit gedurende blootstelling aan hoge concentraties acrylamide en acrylonitril vergeleken met de stabiliteit van het NHase in het ongezuiverde celvrije extract en in de hele cellen.

Tot slot werd de CLEA van het NHase uit *Nitriliruptor alkaliphilus* gecombineerd met de CLEA van een hydroxynitrilase uit *Manihot esculenta* in een eenpots bienzymatische cascadereactie voor de synthese van enantiozuivere alifatische α-hydroxycarboxylamides uit aldehyden in **Hoofdstuk 6**. Stabiliteitstesten wezen uit dat de NHase CLEA's gevoelig waren voor niet wateroplosbare organische oplosmiddelen alsmede voor aldehyden en waterstofcyanide (HCN), maar verassend stabiel en actief waren in een zure waterige omgeving met pH 4-5. De cascadereacties werden vervolgens uitgevoerd met een deelsgewijze toevoeging van HCN en een gematigde concentratie aldehyde in zure waterige buffer met als doel om de ongekatalyseerde achtergrondsreactie te onderdrukken. Na optimalisatie werd deze methode gebruikt om vijf verschillende soorten alifatische α-hydroxycarboxylamides te produceren uit de overeenkomstige aldehyden met goede

opbrengsten en met enantiozuiverheden die vergelijkbaar waren met de enantiozuiverheden verkregen voor α-hydroxynitrillen in hydrocyanering met gebruik van een hydroxynitrilase en een overmaat HCN in organisch oplosmiddel met een minimale (<1%) bufferfase.

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Roger, ik wilde je graag bedanken voor de mogelijkheid om mijn promotieonderzoek in jouw groep te kunnen uitvoeren. Ik wilde je ook bedanken voor al je advies en de mooie en interessante verhalen uit de industrie tijdens de werkbesprekingen. Fred, ik wil jou als mijn dagelijkse begeleider bijzonder bedanken voor alle advies en wijze raad op zowel chemisch, technisch als organisatorisch gebied. Je wist me altijd weer te verbazen met de hoeveelheid (niet-)chemische kennis en droge humor die je paraat had tijdens discussies aan de, nu helaas opgeheven, koffietafel in de Böesekenzaal. Wat ik heel prettig vond was dat je een begeleider was waar ik ook met puur praktische problemen terecht kon. Isabel, je hebt in 2007 het vaandel van Roger als professor van BOC overgenomen. Behalve een goede wetenschapper, ben je ook een goede manager en ik denk dat de toekomst van BOC bij jou in goede handen is. Mieke, wat er van BOC terecht zou komen als jij er niet was weet ik niet. Wat ik wel weet is dat veel dingen in de soep zouden lopen. Bedankt ook voor alle steun die je me hebt gegeven in moeilijke tijden. Je bent een engel! Linda, bedankt voor al je hulp bij het zuiveren en de biochemie. Het volledige gen van het nitrilhydratase uit *Nitriliruptor alkaliphilus* weet tot nu toe steeds door onze vingers te glippen, maar ik weet zeker dat dit van korte duur is. Je enthousiasme en energie hebben mij altijd gemotiveerd en ik wens je een mooie wetenschappelijke carrière toe. Remco, bedankt voor alle technische ondersteuning maar vooral ook voor het lachen tijdens de practica en de koffiepauzes. Nee, ik ga geen Diablo of Guildwars meer spelen, maar ik kan in de toekomst nog wel een keer een spelertje van je kopen. Maarten, bedankt voor alle technische ondersteuning met ons lichtelijk verouderde HPLC park. Ik hoop dat je ooit de apparatuur kan aanschaffen die je graag zou willen hebben. Ik wilde ook graag de rest van de vaste

staf bedanken voor interessante discussies en wetenschappelijke input. Ulf, Leendert, Frank, Joop en Kristina, bedankt!

Furthermore, I would like to express all my gratitude to Dimitry Sorokin. We came into contact exactly at the right moment. I needed nitrile hydratases and you were interested in studying the microbial degradation of nitriles in haloalkaliphilic natural environments. I think that a microbiologist working together with a chemist is a winning combination. The success of this combination is quite clear considering the amount of work we managed to do together. I would also like to express my gratitude to the participants of the COST working group D25/0002/02 on nitrile- and amide-hydrolysing enzymes as tools in organic chemistry and of COST action CM0701 working group 3 on cascade reactions on the nitrile group, especially to Andreas Stolz, Dean Brady, and David Kubáč for fruitful discussions. I would also like to thank Jarle Holt. I think we had an excellent idea to try nitrile hydratases on your tertiary nitrile compounds. It did not work out exactly the way we planned but that is also a considerable part of life as a scientist. I am also grateful to the people from NZomics, in particular Justin Perry, for providing me with several purified enantioselective nitrile hydratases and for interesting scientific e-mail conversations.

A lot of gratitude and respect for my French first year master students from Ecole normale supérieure de Cachan (ENS Cachan). Sandrine, you did a lot of work on the characterisation of nitrile hydratase CLEAs, which eventually resulted in a nice publication in Green Chemistry. Marion, your work on CLEAs and on developing a protocol for nitrile hydratase purification was a great contribution to this thesis. Both of you had a high scientific level for first year master students and it was a pleasure to supervise you. It was truly a pity that you could only work here for three months! Ronald, je hebt samen met Linda hard gewerkt aan het karakteriseren van het nitrilhydratase uit *Nitriliruptor alkaliphilus.* Ik hoop dat je alle persoonlijke problemen kunt overwinnen en je masterproject alsnog af kunt maken.

Ik wil uiteraard verder iedereen bedanken die in de afgelopen 4-5 jaar heeft rondgelopen bij de sectie Biocatalysis and Organic Chemistry voor alle

samenwerking en plezier binnen en buiten werktijden. Natuurlijk moet ik uitdrukkelijk mijn dank uiten aan de mannen met wie ik jaren in de stoffige bibliotheek heb doorgebracht. John, jongen, zonder jou waren deze vier jaar lang niet zo leuk geweest! We hebben samen veel ondernomen, waaronder het organiseren van de BOC studiereis naar Duitsland, Denemarken en Zweden en een mooie week in New York. Ik denk dat ik met alle eerlijkheid kan zeggen dat je de enige man bent met wie ik een week in één bed heb geslapen. Andrzej, you were both a good friend and a good colleague. Since we were working on similar projects I think we were able to help each other a lot. You were my roommate on a lot of conferences as well as travels. If anyone would know why your nickname was Andrzej "The Nighttrain" Chmura, it is me. I would also like to thank Dani a.k.a. Bokito for all the fun in the lab and for keeping me sharp by constantly putting water on my chair. Hans-Peter, zonder jouw sarcasme en vriendschap was deze promotieperiode niet hetzelfde geweest. Ik zou ook graag de mannen van CLEA Technologies willen bedanken. Michiel, Menno, bedankt voor al de humor en wetenschappelijk geblaat tijdens vele koffiepauzes. Ik vind het fijn om jullie nu als collega te hebben. Chrétien, bedankt voor het hosten van de vele game avonden en voor de spoedcursus klagen aan het begin van mijn promotie. Furthermore I would like to thank the following people: Marco N., Marco C., Aleksandra, Hilda, Inga, Chris, Pedro, Monica, Seda, Maria, Luigi, Christophe, Daniel, Luuk, Lars, Silvia, Matthieu, Mapi, David, Florian, Steffi, Marina, Ksenia, Bruno, Aida, Ron, Katya, Serena, Adeline, Karin, Tobias, Selvedin, Jin, Franja, Laura, Federico, Sanjib, Betti, Ivo, Justin, Anne, Myriam, Atsushi, Hector, Jeroen, and others I might forget to mention here. I could write a story about all of you but then the acknowledgements would become bigger than the thesis itself.

I would also like to thank Carol, Joost, Jean-Paul, Rutger, Zheng, and Yang for all the fun we had organising the Biotechnology Study Tour to China. I think we can easily conclude that this study tour was a big success. The only downside is that I still miss the Chinese food.

Natuurlijk zijn er ook veel mensen die ik moet bedanken buiten het wetenschappelijke wereldje. David, Bernard, Roderik, Job, Veronique, bedankt voor

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Uiteraard ben ik mijn hele familie, in het bijzonder mijn ouders en zusje, veel dank verschuldigd voor al hun onvoorwaardelijke steun en interesse gedurende het gehele universitaire traject. De Van Pelt fietsploeg begint vanwege de uiteen liggende woonplaatsen een beetje uit elkaar te vallen, maar ik hoop dat we in de weekenden nog mooie fietstochten op de racefiets kunnen maken. Sono anche molto grato alla mia famiglia italiana Fiorella, Gianfranco, Daniele e Stefania, sebbene ci sia un piccolo problema con la lingua mi avete sempre fatto sentire a casa e parte della famiglia. Last but definitely not least, I would like to thank Daniela. Without you I would not have survived this last year. I think we have shown to be an excellent team in both good and bad times. Ti amo luce dei miei occhi!

ALLEN BEDANKT!

Sander

List of Publications

1. D.Y. Sorokin, **S. van Pelt**, T.P. Tourova, S. Takaichi, G. Muyzer. Acetonitrile degradation under haloalkaline conditions by *Natronocella acetinitrilica* gen. nov., sp. nov., *Microbiology*, 2007, 153, 1157-1164.

2. D.Y. Sorokin, **S. van Pelt**, T.P. Tourova, G. Muyzer. Microbial isobutyronitrile utilization under haloalkaline conditions, *Applied and Environmental Microbiology*, 2007, 73, 5574-5579.

3. D. Kubáč, O. Kaplan, V. Elišáková, M. Pátek, V. Vejvoda, K. Slámová, A. Tóthová, M. Lemaire, E. Galienne, S. Lutz-Wahl, L. Fischer, M. Kuzma, H. Pelantová, **S. van Pelt**, J. Bolte, V. Křen, L. Martínková. Biotransformation of nitriles to amides using soluble and immobilized nitrile hydratase from *Rhodococcus erythropolis* A4. *Journal of Molecular Catalysis B: Enzymatic,* 2008, 50, 107-113.

4. **S. van Pelt**, S. Quignard, D. Kubáč, D.Y. Sorokin, F. van Rantwijk, R.A. Sheldon. Nitrile hydratase CLEAs: The immobilization and stabilization of an industrially important enzyme, *Green Chemistry*, 2008, 10, 395-400.

5. **S. van Pelt**, F. van Rantwijk, R.A. Sheldon. Nitrile hydratases in synthesis, *Chimica Oggi*, 2008, 26 (Suppl.), 2-4.

6. A. Chmura, A.A. Shapovalova, **S. van Pelt**, F. van Rantwijk, T.P. Tourova, G. Muyzer, D.Y. Sorokin. Utilization of arylaliphatic nitriles by haloalkaliphilic *Halomonas nitrilicus* sp. nov. isolated from soda soils, *Applied Microbiology and Biotechnology*, 2008, 81, 371-378.

7. D.Y. Sorokin, **S. van Pelt**, T.P. Tourova. Utilization of aliphatic nitriles under haloalkaline conditions by *Bacillus alkalinitrilicus* sp. nov. isolated from soda solonchak soil, *FEMS Microbiology Letters,* 2008, 288, 235-240.

8. D.Y. Sorokin, **S. van Pelt**, T.P. Tourova, L.I. Evtushenko. *Nitriliruptor alkaliphilus gen. nov., sp. nov.,* a deep-lineage haloalkaliphilic actinobacterium from soda lakes capable of growth on aliphatic nitriles and proposal of *Nitriliruptoraceae* fam. nov. and *Nitriliruptorales* ord. nov., *International Journal of Systematic and Evolutionary Microbiology*, 2009, 59, 248-253.

9. **S. van Pelt**, F. van Rantwijk, R.A. Sheldon. Synthesis of aliphatic *(S)*-α-hydroxycarboxylic amides using a one-pot bienzymatic cascade of immobilised oxynitrilase and nitrile hydratase, *Advanced Synthesis and Catalysis,* 2009, 351, 397-404.

10. **S. van Pelt** et al. Probing the enantioselectivity of a novel group of purified nitrile hydratases, **submitted to Advanced Synthesis and Catalysis**.

11. **S. van Pelt** et al. Characterisation of the cobalt type NHase from *Nitriliruptor alkaliphilus*, **in preparation**.

Oral presentations

1. COST WG D25 meeting on nitrile and amide hydrolysing enzymes as tools in organic chemistry, Graz, April 2006
2. Netherlands Catalysis and Chemistry Conference (NCCC) VIII, Noordwijkerhout, March 2007
3. Minisymposium on biocatalysis at Novozymes, Copenhagen Denmark, June 2007
4. International Conference of Catalysis Applied to Fine Chemistry (CAFC) 8, Verbania Italy, September 2007
5. NIOK Day on Biocatalysis, Delft, September 2007
6. Minisymposium on biotechnology at Jiangnan University, Wuxi China, May 2008
7. Minisymposium on biotechnology at Tsinghua University, Beijing China, May 2008
8. NWO congress for the study groups Design and Synthesis, Structure and Reactivity, Biomolecular Chemistry, Lunteren, October 2008
9. COST WG 3 meeting on cascade reactions on the nitrile group, Prague, May 2009

Curriculum Vitae

Sander van Pelt werd op 2 december 1978 geboren te Rotterdam. In 1997 behaalde hij het atheneumdiploma aan het Libanon Lyceum te Rotterdam. In het jaar 1997 werd begonnen aan de opleiding Scheikundige Technologie en Bioprocestechnologie aan de Technische Universiteit Delft, waar werd gekozen voor het master of science programma Bioprocestechnologie. Deze studie werd *Cum Laude* afgerond met een onderzoek naar de directed evolution van D-2-Deoxyribose-5-Phosphate Aldolase (DERA) in de sectie Biokatalyse en Organische Chemie onder begeleiding van prof. dr. R.A. Sheldon. Tijdens zijn master of science opleiding was hij ook werkzaam bij Evonik, in Hanau, Duitsland en Codexis, in Redwood City, Amerika. Vanaf september 2005 werd een promotieonderzoek verricht in dezelfde sectie onder begeleiding van dr. ir. F. van Rantwijk en prof. dr. R.A. Sheldon. De resultaten van dit onderzoek zijn in dit proefschrift beschreven. Sinds 1 januari 2010 is hij werkzaam bij CLEA Technologies te Delft.

Propositions belonging to the thesis

The Application of Nitrile Hydratases in Organic Synthesis

Sander van Pelt

1. The significant increase in storage stability of cell-free nitrile hydratase as a cross-linked enzyme aggregate indicates that nitrile hydratase deactivation is strongly related to subunit dissociation.
   [This thesis]

2. A green chemist should not go to work by car.

3. The scarcity of enantioselective nitrile hydratases described in literature is related to the relatively small number of chiral nitriles tested.
   [Song et al., Biotechnol. J. 2007, 2, 717-724]

4. The lack of attention to testicular cancer prevention in Holland is unbelievable in view of the fact that it is the most common form of cancer in men aged between 15 and 40 and a highly curable one (99 %) when detected at an early stage.
   [Stichting Kernzaak, kwaliteitscriteria zaadbalkanker, December 2009]

5. The poor applicability of the bi-steady state model for the deactivation of nitrile hydratase in free resting cells at high acrylamide concentrations in a membrane bioreactor is most likely related to cell wall stability.
   [Xudong et al., Chin. J. Chem. Eng. 2009, 17(5), 822]

6. The main function of different journal reference formats is one of distinction and not convenience.

7. By filtering the reaction mixture, Netto *et al*. do not convince the reader of the ease of the recovery of *Candida antarctica* lipase immobilised on magnetic nanoparticles using an external magnetic field.
   [Netto et al., Tetrahedron: Asymmetry 2009, 20, 2299]

8. The main limitation in the application of biocatalysis in industrial synthetic chemistry lies in the lack of reliable commercial availability of most of the enzymes in large quantities.

9. The catalytic promiscuity of enzymes should not be investigated by the direct use of commercial enzyme preparations.
   [Feng et al., Green Chem. 2009, 11, 1933]
   [Duarte et al., Biotechnol. Lett. 2000, 22, 1811]

10. The scope of the Spherezyme method of enzyme self-immobilisation is likely to be limited to enzymes that are highly stable.
    [Brady et al., Biotechnol. Lett. 2009, 31, 1639]
    [Brady et al., BMC Biotechnology 2008, 8:8]

11. The most underestimated and time consuming part of a PhD in biocatalysis is the development of (chiral) analysis methods.

12. The beneficial effect of pressure on the yield of covalent immobilisation can be ascribed to improved contact between the hydrophobic, activated polystyrene surface and the aqueous buffer phase.
    [Kannoujia et al., Biochem. Eng. J. 2009, 48, 136]

These propositions are considered opposable and defendable and as such have been approved by the supervisor prof. dr. R.A. Sheldon.

Stellingen behorende bij het proefschrift

The Application of Nitrile Hydratases in Organic Synthesis

Sander van Pelt

1. De significante verbetering in opslagstabiliteit van het celvrije nitrilhydratase als een gecrosslinked enzymaggregaat wijst op een sterke relatie tussen de dissociatie van de nitrilhydratase subunits en desactivering.
   [Dit proefschrift]

2. Een “groen” chemicus zou niet naar het werk moeten gaan met de auto.

3. Het gebrek aan in de literatuur beschreven enantioselectieve nitrilhydratases is gerelateerd aan de relatief kleine hoeveelheid chirale nitrillen dat getest is.
   [Song et al., Biotechnol. J. 2007, 2, 717-724]

4. Het gebrek aan aandacht voor zaadbalkankerpreventie in Nederland is ongelooflijk wanneer men bedenkt dat het de meest voorkomende vorm van kanker is bij mannen tussen de 15 en 40 jaar met een heel hoog genezingspercentage (99 %) wanneer de ziekte in een vroeg stadium ontdekt wordt.
   [Stichting Kernzaak, kwaliteitscriteria zaadbalkanker, december 2009]

5. De gebrekkige toepasbaarheid van het bi-steady state model voor de desactivering van nitrilhydratase in vrije rustende cellen bij hoge acrylamideconcentraties in een membraanbioreactor is zeer waarschijnlijk gerelateerd aan de stabiliteit van de celwand.
   [Xudong et al., Chin. J. Chem. Eng. 2009, 17(5), 822]

6. Onderscheiding en niet gemak is de voornaamste functie van de verschillen in opmaak voor verwijzingen in wetenschappelijke tijdschriften.

7. Door het reactiemengsel te filtreren overtuigen Netto *et al*. de lezer niet van het gemak van de herwinning van geïmmobiliseerd *Candida antarctica* lipase op magnetische nanodeeltjes door het gebruik van een extern magnetisch veld.
   [Netto et al., Tetrahedron: Asymmetry 2009, 20, 2299]

8. De voornaamste beperking voor het toepassen van biokatalyse in de industriële synthetische chemie is het gebrek aan betrouwbare commerciële verkrijgbaarheid van de meeste enzymen in grote hoeveelheden.

9. De katalytische promiscuïteit van enzymen zou niet bestudeerd moeten worden door direct de commerciële enzympreparaten te gebruiken.
   [Feng et al., Green Chem. 2009, 11, 1933]
   [Duarte et al., Biotechnol. Lett. 2000, 22, 1811]

10. De toepasbaarheid van de Spherezyme zelfimmobilisatiemethode is waarschijnlijk gelimiteerd tot enzymen die zeer stabiel zijn.
    [Brady et al., Biotechnol. Lett. 2009, 31, 1639]
    [Brady et al., BMC Biotechnology 2008, 8:8]

11. Het meest onderschatte en tijdrovende onderdeel van een promotie in de biokatalyse is de ontwikkeling van (chirale) analysemethoden.

12. Het positieve effect van druk op de opbrengst bij covalente immobilisatie kan worden toegekend aan een verbeterd contact tussen het hydrofobe, geactiveerde polystyreenoppervlak en de waterige buffer fase.
    [Kannoujia et al., Biochem. Eng. J. 2009, 48, 136]

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