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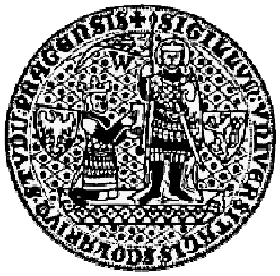

A thesis submitted for the degree of Doctor of Philosophy

Nitrile-hydrolysing enzymes: Fungal nitrilases

Vojtěch Vejvoda

Supervisor: Ing. Ludmila Martínková, CSc.
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Prague 2010

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Signature

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Abbreviations

BSA – bovine serum albumin

CD – circular dichroism

CSMR – continuous stirred membrane reactor

DAD – diode array detector

HPLC – high performance liquid chromatography

SDS-PAGE – sodium dodecylsulfate polyacrylamide gel electrophoresis

Contents

1 INTRODUCTION

Nitriles are widespread in the environment - not only as the natural products of plants but also as the secondary metabolites of microorganisms or higher organisms. Synthetic nitriles and inorganic cyanides occur as pollutants from human activities (industrial wastes, herbicides, etc.). Both natural and man-made nitriles are poisonous to many organisms, the natural ones serving as a protection against enemies (e.g. herbivores). Their toxic activity is used in some herbicides (benzonitrile analogues).

However, all of these compounds may exert negative effects on other beings living in the particular environment and create selection pressures, probably leading to the evolution of enzymes catalyzing the detoxification of nitriles, or, even better, utilizing them as the source of energy and nutrients. Nitrile-metabolizing enzymes (Fig. 1) are relatively rare in organisms, occurring in specific taxons of both prokaryots (nitrilases, nitrile hydratasesChyba! Chybný odkaz na záložku.) and eukaryots (nitrilases).

The ability of the key nitrile metabolism enzymes to also accept synthetic compounds as substrates has provoked great interest in their use as catalysts for nitrile hydrolysis, an approach frequently used for synthetic purposes. The physiological conditions under which these enzymes operate represent a significant advantage compared to chemical catalysts, which usually require drastic conditions. Research into these enzymes has been supported by the success of the first biocatalytic production of a bulk chemical – acrylamide – established in the 1980s in Japan (now producing more than 30 000 ton per year for Mitsubishi Rayon, Co. (Liese et al., 2000). Another promising use of these enzymes is the bioremediation of toxic waste originating from the industrial and agricultural use of nitriles.

In this work we examined several new microbial enzymes catalyzing the degradation of nitriles, with the aim of providing improved tools for organic synthesis, or for the detoxification of waste.

As much more is known about nitrilases in bacteria than in higher organisms such as fungi, we have primarily chosen the latter group of organisms to broaden our knowledge of these enzymes and to find new biocatalysts with potentially better catalytic properties among these less explored enzymes. Genome mining indicated a frequent

occurrence of nitrilases mainly in the *Aspergillus* genus and the anamorph-telemorph pair *Fusarium* - *Gibberella*. In this study the focus was on the *Fusarium solani* species, its potential to produce nitrilase activity and the structural and catalytic properties of this enzyme. The evaluation of a number of techniques, which may be appropriate for the immobilization of nitrilases as unstable enzymes, was also a part of this research.

The following part of the work was concerned with revealing new properties of the alternative nitrile-hydrolyzing enzyme system composed of nitrile hydratase and amidase. This part of the work used a strain of *Rhodococcus erythropolis* which previously proved in our hands to be a powerful nitrile-transforming organism with a number of synthetic applications. A new amidase expressed in this strain was described and its synthetic potential, namely in acyl-transfer reactions, was exploited. New substrates of the nitrile hydratase/amidase enzymes system (dinitriles) have also been discovered. Nitrilase and amidase were applied in cascade systems, in order to increase the purity of biotransformation product (acid) by side product (amide) removal.

Last but not least, attention was paid to the development of methods suitable for an easy, fast and reliable determination of nitrile-transforming activities. The most suitable methods have already been applied to the solution of the above tasks within this work.

Based on the significant amount of data obtained by examining both prokaryotic and eukaryotic enzymes from different perspectives, we were able to draw conclusions on their pros and cons, their potential utility for practical application in laboratory and industrial practice and on the room for biocatalyst improvement.

The text in this work will serve as the introduction for published works, highlight the most relevant results or provide additional information not presented in the articles. For more detailed information, especially on experimental details and other results (purification tables, electromicrographs, etc.), please see the attached articles in the Appendix section.

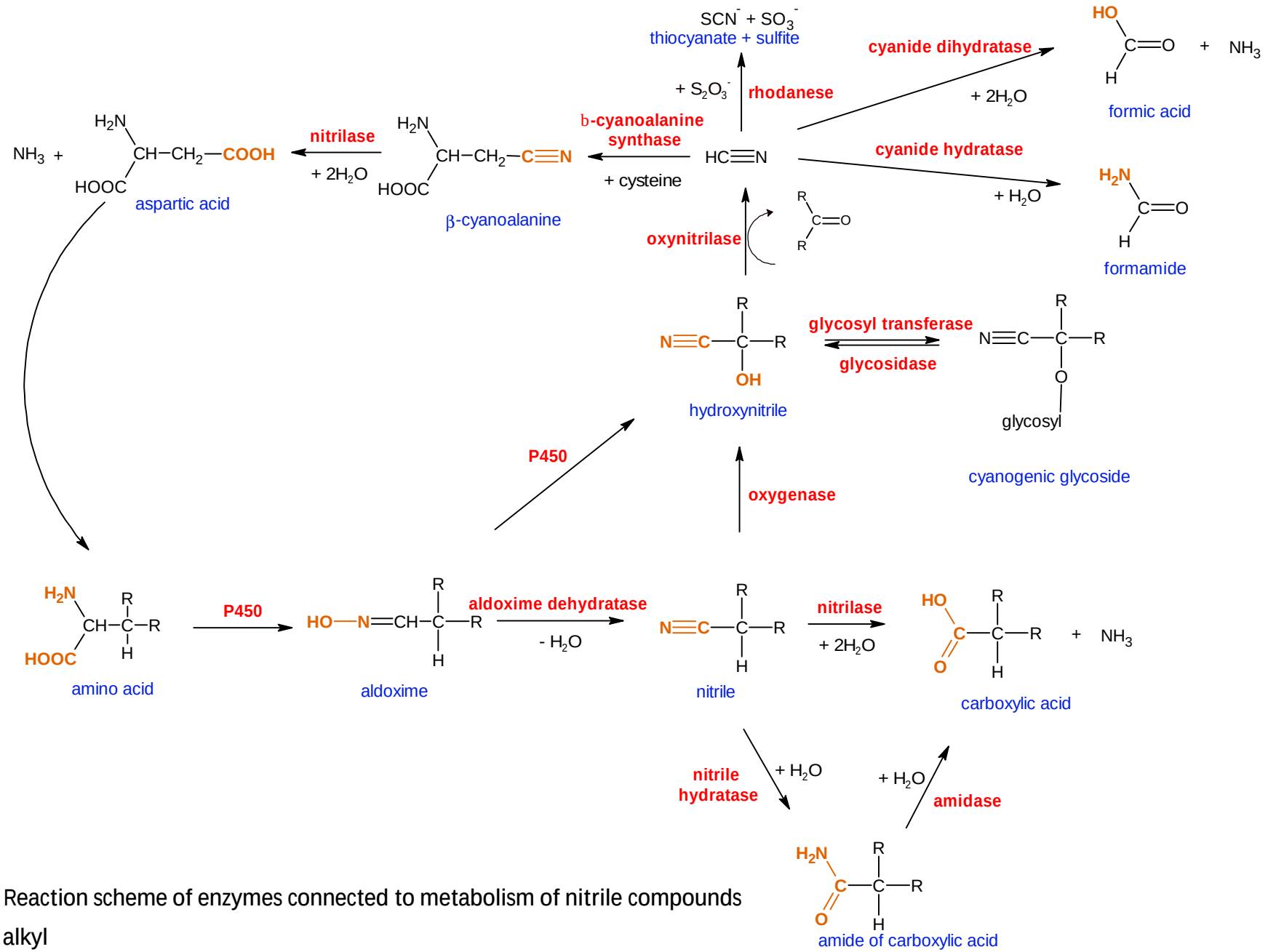

Figure 1 Reaction scheme of enzymes connected to metabolism of nitrile compounds

R – H or alkyl

2 NITRILES OCCURRING IN NATURE

The main sources of natural nitriles (organic cyanides) are plants, in which these compounds are distributed as free nitriles or as conjugates with sugars (cyanoglycosides or cyanoglucosinolates) or with lipids.

The majority of plant cyanogens originate from five amino acids (tyrosine, phenylalanine, valine, isoleucine, leucine), from non-protein amino acids (2-(2-cyclopentenyl)glycine) or from nicotinic acid (Singh, 1998). Amino acids are transformed to nitriles via the intermediate product aldoxime (see Fig. 1Charles

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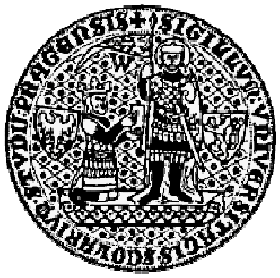

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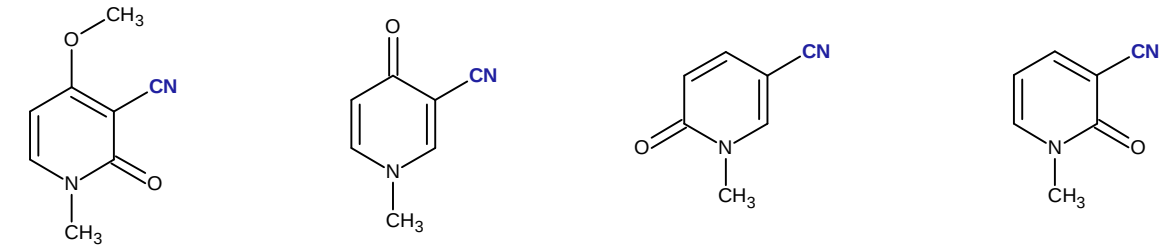
). This reaction is catalyzed by the enzymatic complex of cytochrome P450.

In 2500 plant species more than 60 different cyanoglycosides were found, which fulfill two functions. The first is defence against herbivores and other enemies, and the second is the storage of reduced nitrogen (Zagrobelny et al., 2008). Cyanogenic compounds may also play the role of pheromones, which control mating behaviour (Seidelmann et al., 2003).

Cyanogenic compounds are responsible for the bitter taste of plant products, which is the first defensive effect of cyanoglycosides, while the second, the stronger, is based on the release of hydrogen cyanide by specific enzymes (e.g. oxynitrilases) in the damaged tissues, this discouraging the aggressor and finally being capable of causing the death of that organism. When this defensive system was developed in plants, it is not surprising that a detoxification system was developed in plant enemies, especially in pathogens such as fungi. These organisms parasitize on plants and can only survive if they are able to decompose the poisonous cyano compounds to non-toxic compounds such as carboxylic acids (using nitrilases) or amides (using nitrile hydratases), or by using other enzymes (see Fig. 1). Of course detoxification pathways are also present in plants, to protect them against the negative effects of their own toxins.

Some arthropods, e.g. *Harpaphe haydeniana*, also use nitrile compounds for protection against predators, while secreting fluids rich in mandelonitrile and oxynitrilases (Zagrobelny et al., 2008), which release toxic hydrogen cyanide from this nitrile.

Indoleacetic acid – an auxin (plant hormone) – is synthesized from its cyano precursor under catalysis by plant nitrilase. The ability to produce indoleacetic acid from indoleacetonitrile was tested in 29 plant species and activity for this substrate was only observed in 10 plants belonging to the families *Brassicaceae*, *Poaceae* and *Musaceae* (Thimann and Mahadevan, 1964). However, this observation does not correspond to the ubiquitous distribution of nitrilase genes in the plant kingdom (Piotrowski, 2008). This contradiction can be explained by the low activity of known nitrilases toward this substrate. The ability to convert indoleacetonitrile was also observed in fungi in the early 60´s (Hook and Robinson, 1964).



ricinine mallorepine nudiflorin ricinidine

Figure 2 Examples of simple natural nitriles

Ricinine was isolated from *Ricinus communis* and the nitrilase activity toward this nitrile was described by (Hook and Robinson, 1964). All nitriles depicted in Fig. 2 are related to acalyphin (see below) and have their origin in nicotinic acid metabolism.

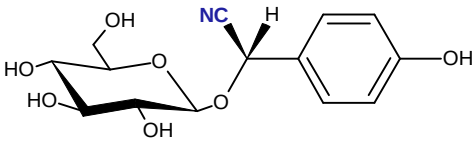
Cyanoglycosides (see Fig. 3) occur in agricultural crops, e.g. manioc (linamarin and lotaustralin), and in the seeds of almonds, peaches, apricots etc. (amygdalin). The high content of linamarin in plants, e.g. manioc, requires special pre-treatment or selective breeding for varieties with lower nitrile content.

Another well-known glycoside is dhurrin, which could account for up to 30% of the dry weight of the leaves of sorghum seedlings. Sambunigrin was found in *Sambucus nigra* (black elder). Acacipetalin and proacacipetalin have only been found in the *Acacia* genus.

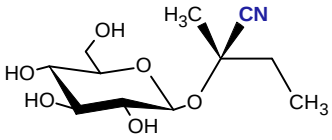
In the bug *Leptocoris isolata,* the cyanogenic glycoside cardiospermin was found along with a mixture of cyanolipids.

Tetraphyllin A is a cyanogenic glycoside originating from the non-protein amino acid 2-(2-cyclopentenyl)glycine and was reported in the family of *Flacourtiaceae* (Singh, 1998) .

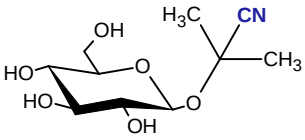
The acalyphin carbon skeleton is a product of nicotinic acid metabolism and was isolated from *Acalypha indica* (Seigler, 1998).



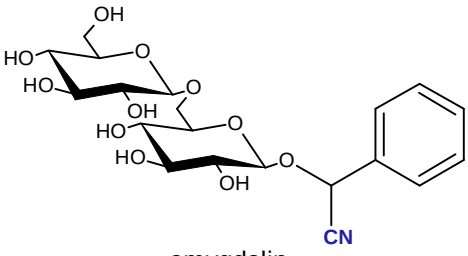
R-dhurrin



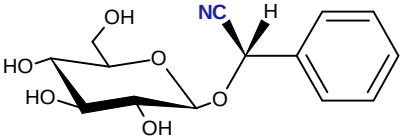
R-lotaustralin



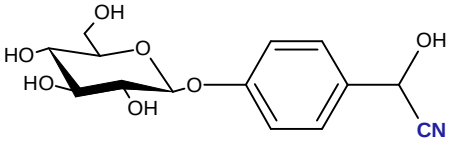
linamarin



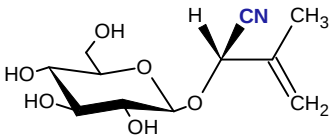
amygdalin



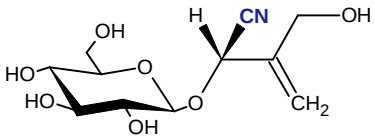
S-sambunigrin



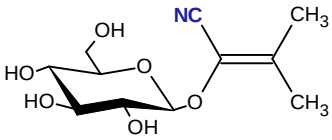
p-glucosylmandelonitrile



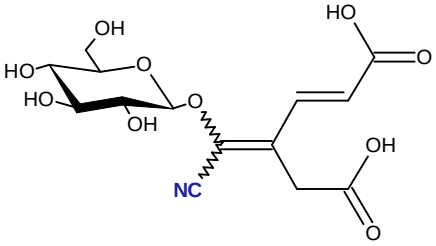
S-proacacipetalin



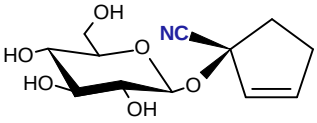
S-cardiospermin



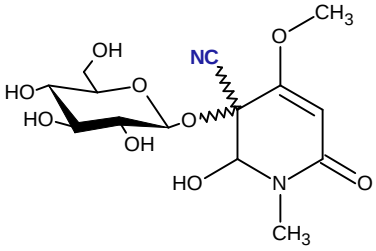
acacipetalin



triglochinin

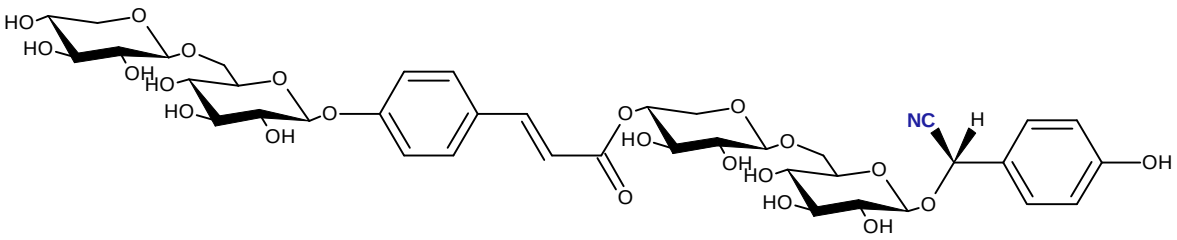


tetraphyllin A



acalyphin

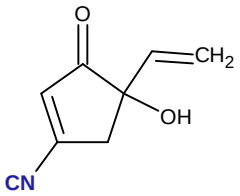
Figure 3 Natural cyanoglycosides occurring mostly in crop-plants (Singh, 1998)



Anthemis glycoside A

Figure 4 Complex natural cyanoglycoside

Other interesting cyanide compounds are fungal or bacterial antibiotics able to inhibit the growth of competitive organisms (Baxter and Cummings, 2006). *Chromobacterium violaceum* ATCC 53434 produces the isonitrile antibiotic aerocyanidin that is active against Gram-positive bacteria and *Trichoderma harzianum* produces homothallin II (see Fig. 5), effective against Gram-positive and Gram-negative bacteria but also against fungi. The production of antibiotics in an environment with limited resources is an advantage for these organisms.



Homothallin II

Figure 5 Fungal isonitrile antibiotic from *Trichoderma harzianum*

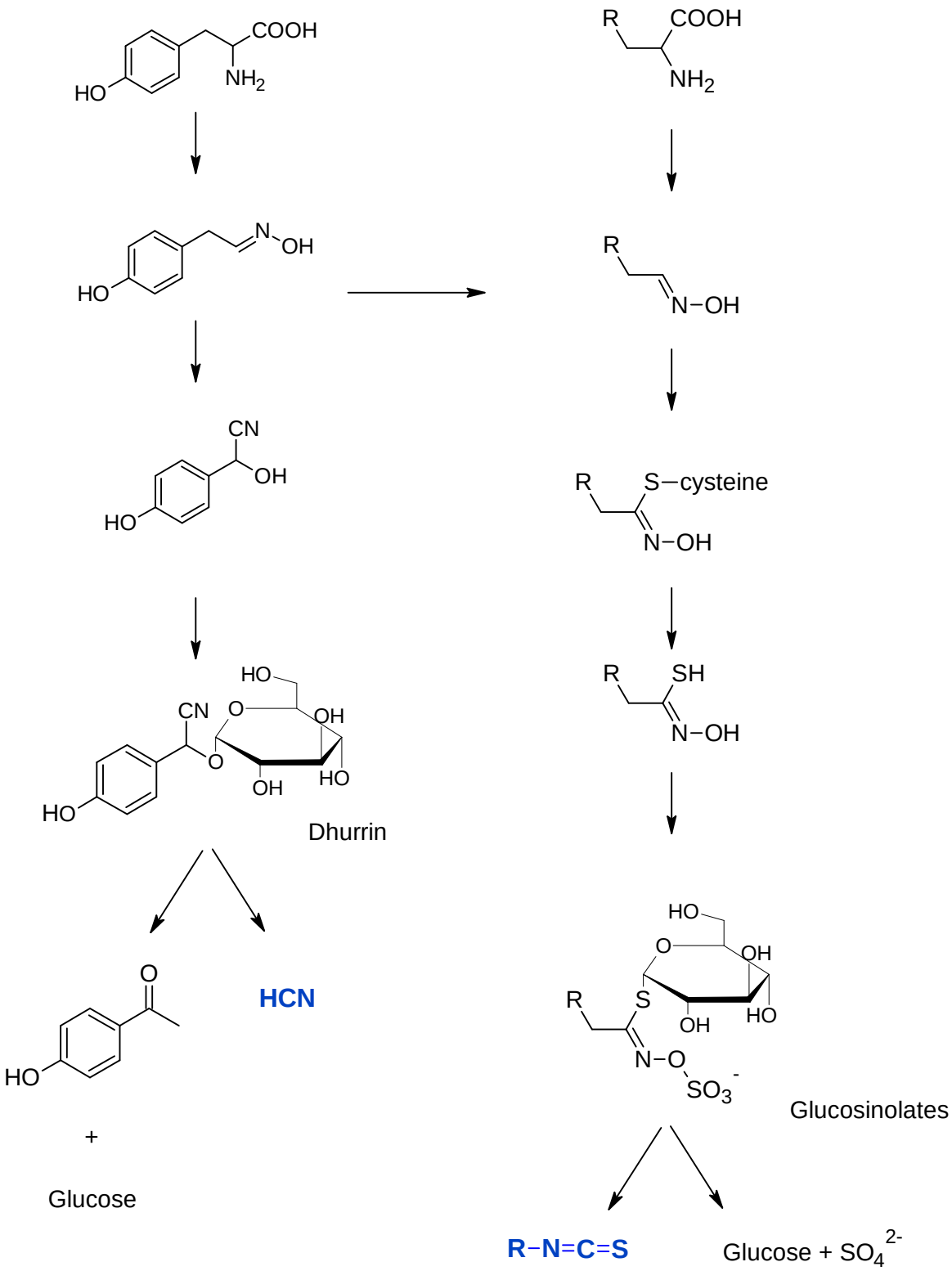


Figure 6 Formation of cyanogenic glycosides and glucosinolates originating from amino acids. If the plant is wounded, toxic hydrogen cyanide is released from cyanoglucosides and poisonous mustard oil from glucosinolates. According to (Zenk and Juenger, 2007)

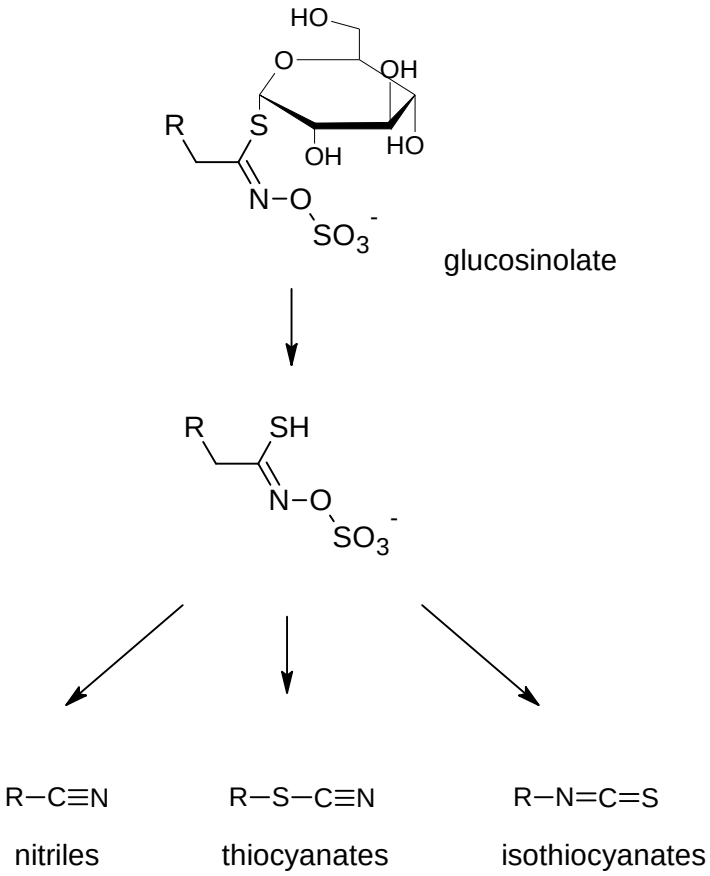


Figure 7 Glucosinolate metabolism in *Brassicales*. After wounding the plant, glucosinolates are degraded by myrosinase and the main product of the reaction is isothiocyanate. Alternatively, nitrile or thiocyanate is formed by the epithiospecifier protein or thiocyanate-forming protein, respectively. According to (Piotrowski, 2008)

Four types of cyanolipids were found in seed oil, which were all derived from leucine. These lipids mainly occur in *Sapindaceae* but also in *Hippocastanaceae* and *Boraginaceae*.

Two types, which have a long-chain fatty acid attached to hydroxynitrile, are cyanogenic as opposed to non-cyanogenic cyanolipids, which are apparent rearrangement products derived from the same precursor as the above-mentioned cyanolipids.

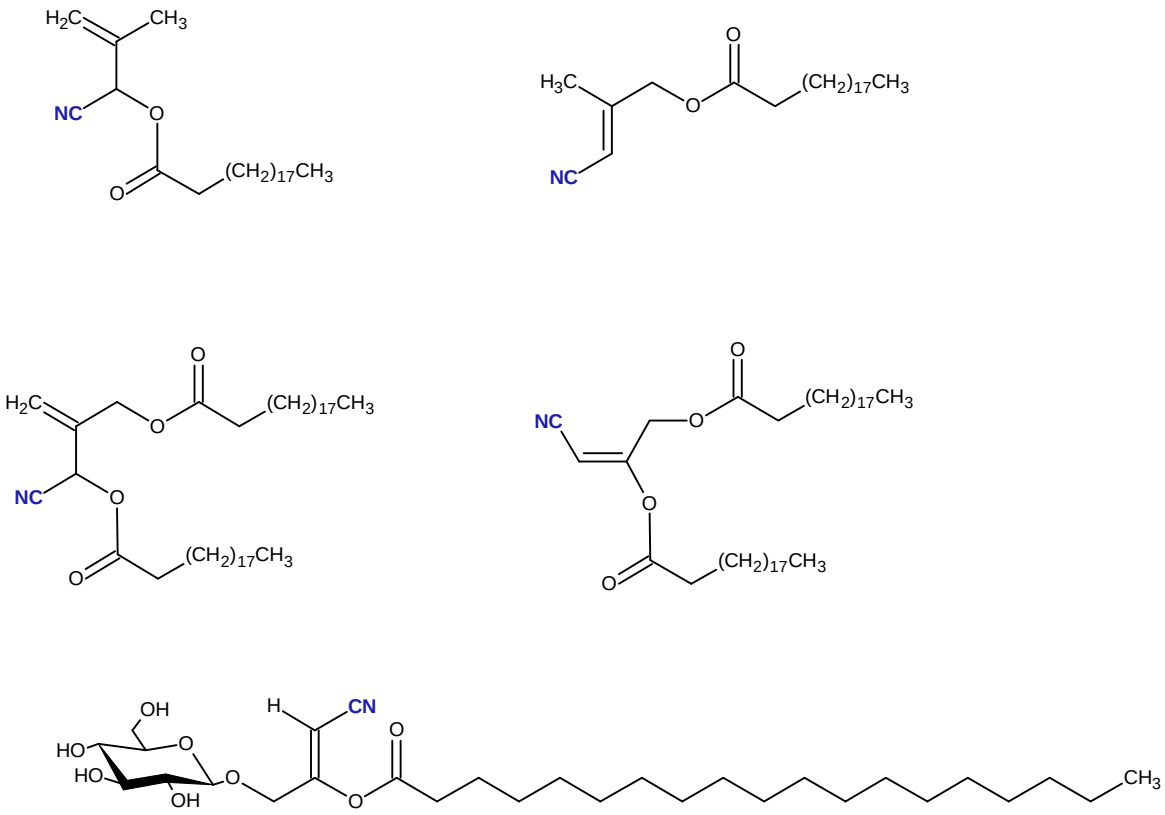


Figure 8 Cyanolipids

The storage function of cyano compounds is also important. Cyanoglycosides serve as a stock of reduced nitrogen and can be used for the synthesis of molecules containing nitrogen in different forms, amino acids, amines, etc. (see Fig. 1).

4 INDUSTRIAL NITRILES

POLLUTANTS

Mankind has influenced the environment for a long time and cyanides have been known for centuries. Inorganic cyanides are used in galvanizing baths and in the mining industry, especially in gold leaching. It is obvious that these toxic substances leak into the environment, forming harsh conditions that are very hostile to living organisms. Only those able to detoxify the cyanide wastes are able to survive in such an environment that is organisms which have developed a defensive system of enzymes (cyanide hydratase or cyanide dihydratase) transforming hydrogen cyanides into less toxic formic acid or formamide. Organisms with this activity were found in gold mines or former gasworks (Barclay et al., 1998).

Cyanide pollution is very significant; in the U.S.A. 18 billion litres of cyanide-containing waste are estimated to be generated annually (ATSDR, 2004; Baxter and Cummings, 2006). Additional sources of cyanide result from food and feed production. For example during cassava starch production large amounts of cyanogenic glycosides are released, leading to cyanide concentrations in wastewater as high as 200 $mg.L^{-1}$ (Baxter and Cummings, 2006).

Organic nitriles have been used, especially in the last century, due to the rise of the chemical industry. The main nitrile pollutants in nature are halogenated pesticides, e.g. bromoxynil, ioxynil, chloroxynil or chlorothalonil (see Fig. 9). Bromoxynil is used as a post-emergent herbicide, chlorothalonil is a broad-spectrum fungicide used as anti-fouling agent on e.g. boat hulls (Baxter and Cummings, 2006).

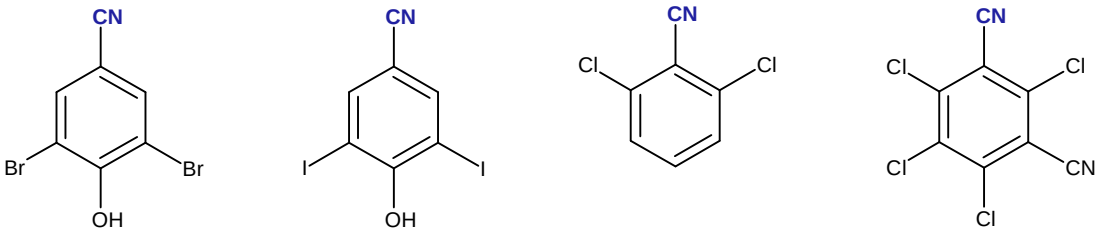
Table 1 Benzonitrile-base agrochemical usage (Baxter and Cummings, 2006)

Compound	Effect	Application	Consumption in USA per year (tons)	Dose ($kg.ha^{-1}$)	LD_{50} ($mg.kg^{-1}$)	Solubility in water ($mg.L^{-1}$)
Dichlobenil	broad spectrum contact herbicide	private gardens, orchards	110-161	4-30	1014-4460	18
Bromoxynil	selective contact herbicides	to control broad-leaved weeds	1800-2200	0.4-1.1	110-260	130
Ioxynil	selective contact herbicides	to control broad-leaved weeds	N/A	0.4-1.1	110-230	50
Chlorothalonil	broad spectrum, non-systematic fungicide	peanuts, potatoes, tomatoes	6000	N/A	10000	600

N/A – not available

An increase in the use of these nitriles followed the restriction of triazine herbicides in many countries, and not surprisingly led to significant pollution of not only surface water but also groundwater. In Denmark, the degradation products of dichlobenil were found in 20 % of the tested wells and in 6 % of them they exceeded the EC threshold of 0.1 $\mu g.L^{-1}$ (Holtze et al., 2008). For additional information see Table 1.

Other nitrile pollutants are organic solvents (acetonitrile) or substances from industrial manufacturing processes (acrylonitrile, cyanopyridine).



bromoxynil ioxynil dichlobenil chlorothalonil

Figure 9 Benzonitrile analogues used as pesticides

MATERIAL FOR ADDED VALUE COMPOUNDS

Enzymatic reactions were found to be useful tools decades ago, and in some cases are the only options when high stereoselectivity is needed.

Despite the fact that many enzymatic reactions are known, only a few of them are used commercially. The main reason is probably the instability of enzymes, variability of enzyme batches, or laborious and/or expensive preparation of the enzyme. Nitrile-converting enzymes are able to replace the nitrile group with a different functional group (carboxylic acid, carboxyl amide) and increase the polarity of the compound. Nitrile hydratases are currently not only employed in some reactions in the laboratory, but also in industry. For the production of acrylamide (used for the manufacture of acrylic resins, enamel coating etc.), the nitrile hydratase from *Rhodococcus rhodochrous* is used at Mitsubishi Rayon on a scale of about 30 000 tons per year (Liese et al., 2000). At the Lonza, the same enzyme is used for the production of nicotinamide from 3-cyanopyridine (Liese et al., 2000).

Another interesting example of the potential usage of the nitrile hydratase from *Rhodococcus* is the production of boron-containing amides (Cowan et al., 1998) or acids, which are analogues of amino acids and possess a variety of biological activities (antineoplastics, antihyperlipidemic, analgetics or antiarthritic agents (see Fig. 10).

Nitrilase activity was first found to act on 3-indoleacetonitrile (a plant hormone). This hydrolase can also be used for the production of another plant hormone, nicotinic acid, which is an important plant growth factor that is a part of commercially available products aimed at root forming in plant slices, and of course an essential vitamin.

A pharmaceutically important product of a nitrilase reaction is (*R*)-3-hydroxy-4-cyanobutanoic acid, which is an intermediate of the cholesterol-lowering drug Lipitrol (Singh et al., 2006).

The immobilized enzyme from *Alcaligenes faecalis* ATCC 8750 was used for stereoselective hydrolysis of mandelonitrile to (*R*)-mandelic acid. This immobilisation technique is useful for the production of hydroxy analogues of methionine derivatives for cattle feeding (Singh et al., 2006). Recently, the heterologously expressed nitrilase from *Pseudomonas fluorescens* EBC191 and mutants of this enzyme proved useful for the production of (*S*)-mandelic acid or (S)-mandelamide from (*S*)-mandelonitrile prepared by using a stereoselective hydroxynitrile lyase (Sosedov et al., 2009).

It was also reported that the bacterial nitrilase from *Rhodococcus butanica* is able to stereoselectively transform a racemic mixture of naproxen to non-steroidal antiflogisticum (*S*)-naproxen in 99% ee (Singh et al., 2006).

Of course, many other nitriles are substrates for nitrilases (for a review see (Martínková et al., 2003; Martínková and Křen, 2010). Fungal nitrilases and their biotechnological potential were recently reviewed by Martínková et al.,(2009a).

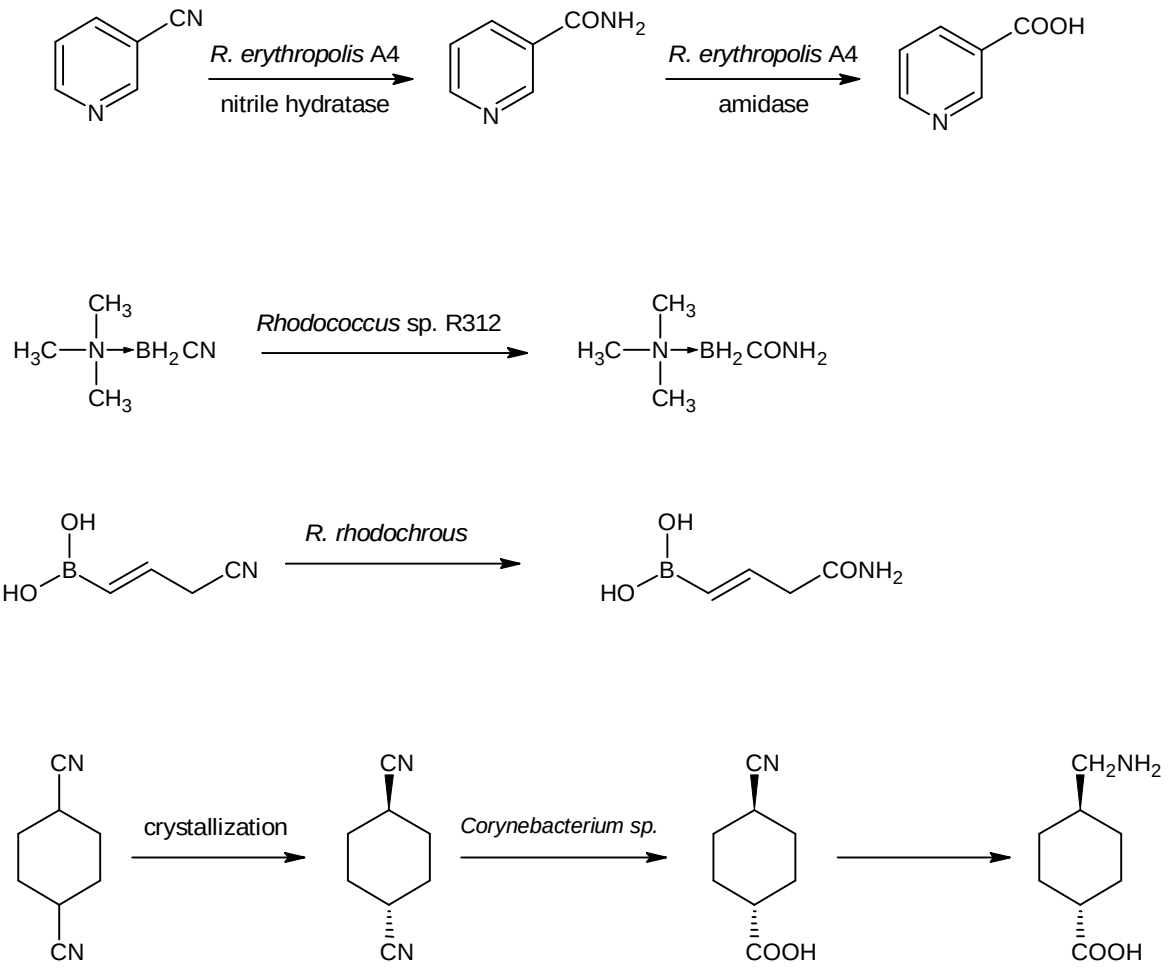


Figure 10 Examples of reactions catalyzed by enzymes of nitrile metabolism

5 NITRILASES

Nitrilases (EC 3.5.5.1) constitute branch 1 of the nitrilase superfamily, which consists of enzymes hydrolyzing non-peptide C-N bonds (Pace and Brenner, 2001). They do not require any co-factors or other additives for their function.

Nitrilases were described for the first time in the 1960s and one of these early works reported the ability of fungi from the genera *Aspergillus*, *Penicillium, Gibberella* and *Fusarium* to convert 3-indoleacetonitrile into the plant hormone 3-indoleacetic acid (Thimann and Mahadevan, 1964).

The first two purified nitrilases came from *Pseudomonas* sp. (Hook and Robinson, 1964) and barley leaves (Thimann and Mahadevan, 1964). Research into nitrilases was intensified in the late 1970s in response to the increasing interest in biotechnologies. New nitrilases were purified and characterized from both prokaryotic and eukaryotic organisms, namely *Nocardia* (*Rhodococcus*) (Harper, 1976) and *Fusarium solani* IMI 196840 (Harper, 1977). The enzyme from the latter organism remained the only characterized fungal nitrilase until the late 1980s, when Goldlust and Bohak (1989) described a nitrilase in *Fusarium oxysporum* f. sp. *melonis.*

Most nitrilases are multimeric enzymes typically consisting of 12 to 16 subunits, the mass of which is approximately 40 kDa.

Knowledge of the structure and function of bacterial nitrilases has substantially improved over the last two decades (for a review, see (Banerjee et al., 2002; O'Reilly and Turner, 2003; Singh et al., 2006). Nowadays it is accepted that the active site of these enzymes consists of the catalytic triad glutamic acid-lysine-cysteine. According to known sequences available in databases, it is obvious that the environment of the active site is conserved in most described nitrilases, and that it is very similar to other enzymes from the nitrilase superfamily (Pace and Brenner, 2001).

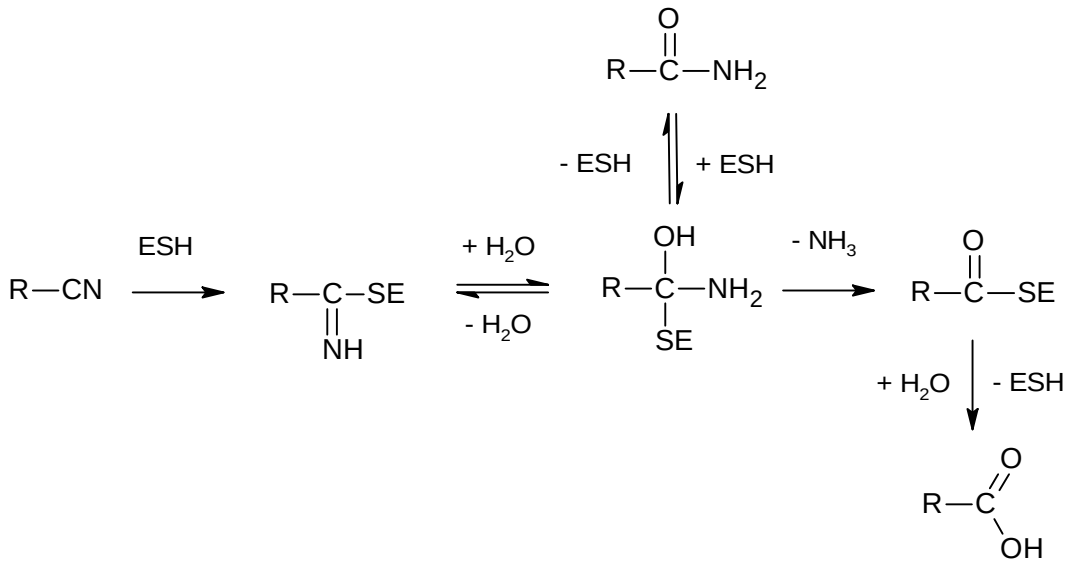


Figure 11 Reaction scheme of nitrile hydrolysis by nitrilase and production of the side product amide (based on (Kobayashi et al., 1998)

The largest group of nitrilases prefers aromatic over aliphatic nitriles, (for reviews, see (O'Reilly and Turner, 2003; Martínková et al., 2009a; Thuku, et al., 2009; Appendix 10). On the other hand, nitrilases operating preferentially on either aliphatic or arylaliphatic substrates have also been described (ibid.).

The reaction mechanism of nitrile transformation to carboxylic acid or amide (as side-product) in Fig. 11 has not been completely confirmed, but is accepted as highly probable. In this enzymatic reaction, an important role is played by sulphydryl group of cystein, which forms a bond to the substrate followed by the addition of water. An alternative breakdown of the enzyme-substrate complex leads to the formation of amide. The ratio between acid and amide depends on the substrate characteristics (bulkiness and electronic effects; Fernandes et al., 2006; Kobayashi et al., 1998).

The lack of determined crystal structure complicates understanding nitrilase function and subunit assembly. On the other hand, using electron microscopy, new possibilities in enzyme structure prediction were found. According to these studies (Thuku et al., 2009) nitrilases and related enzymes (cyanide hydratase, cyanide dihydratase) form spiral-like structures, which are assembled to supramolecular complexes of more than 1 MDa in mass.

SCREENING AND INDUCTION OF NITRILASES

A screening for nitrile-utilising organisms was carried out at the Laboratory of Biotransformation, Institute of Microbiology ASCR, a few years ago (Nikolaou, 2004; Kaplan, 2004). A number of potential nitrilase producers were found, however with low levels of nitrilase activity. Therefore, approx. 20 nitriles were tested as inducers and finally the problem of activity induction was solved by O.Kaplan (see Appendix 3). 2-Cyanopyridine was selected as a very useful inducer for all tested filamentous fungi, increasing the nitrilase production by up to three orders of magnitude.

This new inducer was also used with *Fusarium solani* O1 and approx. 400 U of nitrilase activity per litre of culture was obtained. This enzyme production was more than one hundred times higher than with the originally used inducer, 3-cyanopyridine, but still lower than for *Fusarium solani* f. sp. *melonis* (Goldlust and Bohak, 1989) with a yield of 830 $U.L^{-1}$. A two-step cultivation was used for the latter fungus, including the transfer of the mycelium from a growth-supporting into a nitrilase-inducing medium. Therefore, we used a similar method and, after its optimization, the yield of nitrilase activity was increased to 3000 $U.L^{-1}$ (Fig. 12) (Appendices 2 and 7).

This fact encouraged us to examine another strain of the same organism, in which one of the first nitrilases was described (Harper, 1977), *F. solani* IMI 196840. The above two-step method led to an increase in the originally published activity of 4 $U.L^{-1}$ up to approx. 1500 $U.L^{-1}$ (see Table 2). This was a lower yield than with *F. solani* O1, but still sufficient for straightforward purification, handling and use of the enzyme (Appendix 13).

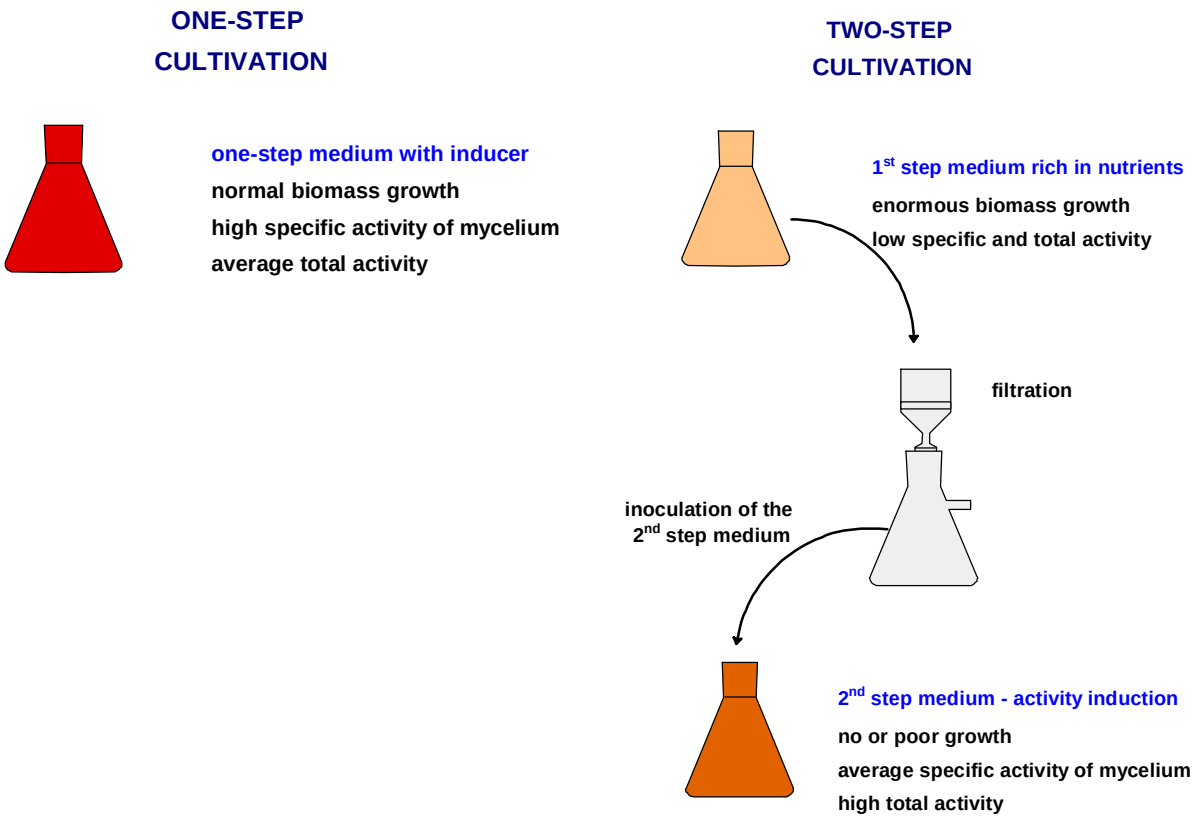


Figure 12 One- and two-step cultivation

The two-step cultivation proved to be beneficial for both of the *Fusarium* genus strains but failed to be with *Aspergillus niger* (Kaplan, *personal communication*). This may be due to the deleterious effect of the change in medium composition or pH on the physiological state of the mycelium, as *Aspergillus* released acids into the medium during cultivation, hence lowering the pH of the growth medium.

Another benefit of *Fusarium* strains is their enzyme stability in the lyophilized mycelium, no activity loss being observed after one year, when the mycelium was stored at -20 °C. As a result, a ready-to-use preparation was available at any time and could be used for purification, reaction or other applications.

Table 2 Different methods of cultivation of *F. solani* strains for nitrilase production

Fusarium solani O1

	Total activity ($U.L^{-1}$)	Increase
original inducer (3-cyanopyridine)	3.2	
2-cyanopyridine as inducer		
one-step cultivation	437	137
two-step cultivation	3000	938

Fusarium solani IMI 196840

	Total activity ($U.L^{-1}$)	Increase
original inducer (benzonitrile)	4	
2-cyanopyridine as inducer		
two-step cultivation	1565	391

NITRILASES FROM *FUSARIUM SOLANI*

5.2.1 Enzyme purification

The same purification protocol could be used for both nitrilases from the *Fusarium* genus, as the enzymes were very similar in their physico-chemical properties.

The powdered lyophilized mycelium was routinely used as the starting material due to its stability (see above) and extracted with TRIS/HCl buffer containing 0.8 M ammonium sulfate. In general, the yield of activity did not fall below 80 % in this step and in the presence of ammonium sulfate, the specific activity of the cell-free extract increased by a factor of at least two (typically from 7 to 15 $U.mg^{-1}_{protein}$) in contrast to the TRIS buffer without this salt. Furthermore, repeated extraction of the pellet after centrifugation made the extraction step nearly quantitative.

Most nitrilases, including all known fungal enzymes, are high molecular weight proteins (typically more than 400 kDa) and therefore the purification method of choice is gel filtration. However, the large volume of cell-free extracts was limiting for this method. This led us to choose Phenyl Sepharose (low substituted) chromatography as the first step, which led to concentration of the enzyme solution (followed by increasing the protein concentration by centrifugation through membrane filters). Gel filtration on Sephacryl S-200 was then applied as the second step. The final step - ion exchange chromatography using a strong anion exchanger Q Sepharose - yielded only two peaks, which were easily separated to give the nitrilase in high purity. This was confirmed by SDS-PAGE and by circular dichroism measurement (for more details see Appendix 13). The purification of nitrilases is summarized in Appendices 7 and 13.

Table 3 Summary of *Fusarium* nitrilase purification

Strain	Purification (fold)	Yield (%)	Specific activity toward benzonitrile (U mg $^{-1}$)
F. solani O1	9.9	25.7	156
F. solani IMI 196840	20.3	26.8	144

Both enzymes were purified with similar yields of ¼ of the initial activity and similar specific activities toward benzonitrile (reference substrate) – i.e. approx. 150 $U.mg^{-1}_{protein}$. This specific activity is very high compared to bacterial nitrilases, but is not exceptional in fungal nitrilases as shown in Table 4. This suggests a significant potential for these enzymes in laboratory and industrial practice.

Table 4 Specific activity of selected nitrilases for benzonitrile

Enzyme	Organism	Specific activity (U mg^{-1} protein) for benzonitrile[a]	Reference
Aromatic nitrilase	*Aspergillus niger* K10	91.6	Appendix 5
	Fusarium solani f. sp. *melonis*	143	(Goldlust and Bohak, 1989)
	Fusarium solani IMI196840	1.66	(Harper, 1977)
	Fusarium solani IMI196840	144	Appendix 13
	Fusarium solani O1	156.0	Appendix 7
	Rhodococcus rhodochrous J1	15.9	(Kobayashi et al., 1989)
Aliphatic nitrilase	*Rhodococcus rhodochrous* K22	0.737	(Kobayashi et al., 1990)

[a] with benzonitrile as substrate except for strain *R. rhodochrous* K22 (with crotononitrile)

5.2.2 Structural properties

Information on the primary structure of the nitrilases from *F. solani* was obtained by Edman degradation (*N*-terminal sequences, peptides obtained on trypsine digestion) and by MALDI (sequences of the internal peptide fragments). On comparison with sequences available in databases, this data allowed us to confirm that the purified enzymes are true nitrilases. Using sequences obtained by MALDI and Edman methods, we performed data mining from sequence databases (http://www.ncbi.nlm.nih.gov) to find the closest relatives of our enzymes. These results can further serve to design primers for selected sequences, to amplify the genes of interest by PCR and to attempt expressing them in active forms.

The selected sequences with highest similarity to those of digests from *F. solani* nitrilases are displayed in Table 5. The nitrilase from *F. solani* IMI 196840 exhibits high

similarities (83 - 99 % of 141 sequenced amino acid residues) with putative nitrilases from *Nectria haematococca, Aspergillus* and *Gibberella* (an anamorph of *Fusarium*) in decreasing order (for details see Appendix 13). Also, the nitrilase from *F. solani* O1, in which, however, only 41 amino acid residues were determined, exhibited a high similarity to the same enzymes. The putative nitrilases with high similarities to enzymes purified by us are listed in Table 5.

Table 5 Hypothetical nitrilases with high amino acid sequence similarity to *Fusarium* nitrilases purified in this work

Organism	Sequence accession number	Percent identity [a]
Nectria haematococca mpVI 77-13-4	EEU37388	100
Aspergillus terreus NIH2624	XP_001209938	69
Aspergillus oryzae RIB40	XP_001824866	74
Nectria haematococca mpVI 77-13-4	EEU37154	68
Nectria haematococca mpVI 77-13-4	EEU36214	67
Gibberella zeae PH-1	XP_386656	66
Gibberella moniliformis	ABF83489	65

[a] Amino acid sequence compared with that of the hypothetical nitrilase from *N. haematococca*

The molecular mass of the subunit, determined by SDS-PAGE, was about 40 kDa for both nitrilases. SDS-PAGE was carried out under reducing and non-reducing conditions, and no differences in the migration of bands were observed for either enzyme, suggesting that there were probably no disulfide bonds in the enzyme molecules.

Far-UV CD spectra (CD) were used to determine and compare the type of secondary structures of the purified nitrilases. These measurements were done as part of a collaborative work at the Loschmidt Laboratory (Masaryk University Brno) under the supervision of Prof. J. Damborský and Dr. R. Chaloupková (February 2008). Data for both

nitrilases from *F. solani* strains and for the recombinant nitrilase from *A. niger* K10 were obtained by this method.

The CD spectra of both *Fusarium* nitrilases showed one positive peak at 195 nm and two negative ones at approx. 221 and 208 nm, which is characteristic for α-helical structures (Appendix 13). Both tested enzymes had a similar intensity of CD spectra, suggesting an almost identical content of their secondary structures. Protein secondary structure prediction based on measured CD data is summarized in Table 6. At the same time, the melting points of the enzymes were also determined and could be compared with other techniques used for describing their thermostability (the melting temperature is the temperature at which half of enzyme is in an active conformation, while the other half is denatured). The nitrilase from *F. solani* IMI 196840 (T_m = 49.3 °C) exhibited a slightly higher thermostability than the nitrilase from *F. solani* O1 (T_m = 47.8 °C). The structural features and melting temperature of the recombinant nitrilase from *A. niger* K10 (Kaplan, *personal communication*) were comparable with those of *F. solani* nitrilases. Both enzymes also exhibited the same shape and intensity of fluorescence spectra (Appendix 13), suggesting that their tertiary structures were almost identical. Apart from this, CD measurement confirmed the low amount of contaminating proteins in the purified enzyme preparations.

Table 6 Secondary structures in nitrilase molecule

Organism	**α**-Helix (%)	**β**-Sheet (%)	Turn (%)	Others (%)	T_m (°C)
F. solani O1	30	20.8	16.4	32,8	47.8
F. solani IMI 196840	30	21	16.4	32.6	49.3
A. niger K10 - recombinant	26.4	23.5	17.4	32.7	45-50

As for their quaternary structures, nitrilases are typically oligomers with a high molecular weight and are near the exclusion limit of columns routinely used for gel filtration. This fact made the determination of molecular weight difficult and less accurate. Nevertheless, using various columns (TSK 3000SW, Superdex 200, Sephacryl S-200) allowed the molecular weight of these enzymes to be determined to be more than 440 kDa. Using electron microscopy (performed by Dr. Oldřich Benada, at the Laboratory of Molecular Structure Characterization, Inst. of Microbiol., ASCR), these nitrilases were

observed to aggregate in supramolecular rods, and this could be the reason for the further complications in determining the molecular mass of the native enzymes.

Recently, several cyanide-converting enzymes such as cyanide dihydratases from *Bacillus pumilu*s and *Pseudomonas stutzeri* (Sewell et al., 2005), cyanide hydratases from *Neurospora crassa* (Dent et al., 2009), *Gloeocercospora sorghi* (Woodward et al., 2008) and the nitrilase from *Rhodococcus rhodochrous* J1 (Thuku et al., 2007), were found to share the ability to assemble into supramolecular helical filaments. These clusters have molecular weights of more than 1500 kDa. This property was revealed by electron microscopy, which is a method of choice for the study of high-molecular weight enzymes.

The electron micrographs of enzymes stained with uranyl acetate illustrate the formation of supramolecular structures (see Appendix 7). The nitrilase from *F. solani* formed helical rods up to 500 nm long or aggregates. In samples of the *A. niger* nitrilase, long rods were also observed, which however differed in their structure and length (up to 250 nm) from those of the *F. solani* enzyme (see Appendix 7). In addition, the enzyme from *A. niger* seemed to form no aggregates. This data is comparable to that obtained for bacterial nitrilases or cyanide (di)hydratases. It is possible that the observed structures are typical for the whole group of cyanide hydrolases.

The lack of crystallography data prevented deep insight being gained into the structure and function of nitrilases, but a considerable homology level between the active sites of these enzymes and proteins whose structure has been resolved, e.g. the NitFhit protein from *Caenorhabditis elegans* (Pace et al., 2000), the *N*-carbamoyl-D-amino acid amidohydrolase from *Agrobacterium* sp. strain KNK712 (Nakai et al., 2000) and the C-N hydrolase from *Saccharomyces cerevisiae* (Kumaran et al., 2003), can be helpful in nitrilase modelling. Together with electron microscopy, the alignment of the nitrilase sequence with those of the crystallized protein allowed the first hypothetical model to be proposed of a nitrilase specific for organic cyanides (from *Rhodococcus rhodochrous* J1 (Thuku et al., 2007)). Our current investigations of the enzymes from filamentous fungi are based on similar methods and recently resulted in the construction of the first model of nitrilase in filamentous fungus (*Aspergillus niger*; Kaplan et al., 2010).

5.2.3 Catalytic properties

As with their structure, both purified enzymes from *F. solani* species are also very similar in their catalytic properties, except for minor differences in substrate specificity.

The highest activity for both enzymes was observed over the range 40 – 50 °C and in slightly alkaline media (pH 8).

Both purified enzymes exhibited a high specific activity towards benzonitrile - 156 and 144 $U.mg^{-1}_{protein}$ for enzymes from *F. solani* O1 and IMI 196840, respectively. This activity is comparable with that in the strain *F. oxysporum* f. sp. *melonis* (Goldlust and Bohak, 1989).

These enzymes can be classified as aromatic nitrilases according to their substrate specificity, but also accept aliphatic nitriles, although with significantly lower activity. Aromatic nitriles with a substituent at position 2 are generally poor substrates, probably due to steric hindrances. 4-Cyanopyridine and, of the substituted aromatics, 3-chlorobenzonitrile are among the best substrates for both enzymes.

A beneficial feature of these nitrilases is the formation of a low amount of amide as by-product. In comparison with the fungal nitrilase from *A. niger* K10, which produced 9 and 34 % amide via the hydrolysis of benzonitrile and 4-cyanopyridine, respectively (see Appendix 5), the nitrilases from *F. solani* formed much less (below 5 %) amide in the total product. The amount of amide as by-product increased at sub-optimal reaction conditions (higher or lower pH, lower temperature).

The ability of the nitrilase from *A. niger* K10 and *F. solani* O1 to accept branched and substituted amino nitriles was described by ourselves (see Appendix 9). The enzymes exhibited slightly differing substrate specificities and were both different from the commercially available nitrilases from Biocatalytics, Inc. (now Codexis), which have been well documented to act on cycloaliphatic, aliphatic and arylaliphatic nitriles.

The fungal enzymes are able to discriminate between the *cis*- and *trans*-isomers of γ-amino nitriles. This phenomenon can be potentially used to separate the respective isomer mixtures. On the other hand, the enantioselectivity of the fungal enzymes was only moderate.

The ability of the nitrilase from *F. solani* O1 to accept substrates in concentrations higher than those usually used for activity determination (25 mM) was also examined, in

order to test the potential of this enzyme for preparative conversions. These tests were successful with 3- and 4-cyanopyridine, total conversions having been achieved for 300 and 500 mM of the substrates, respectively. Similar results were obtained for the nitrilase from *F. solani* IMI 196840. Benzonitrile could be also almost quantitatively converted at concentrations up to 500 mM.

The problem of many nitrile substrates is their low solubility in the aqueous reaction mixture and it is sometimes difficult to obtain the desired substrate concentration (usually 10 – 25 mM). To improve solubility, co-solvents such as lower alcohols (methanol), dimethylsulfoxide etc. are usually added to the reaction mixtures.

In this work, methanol was the common solvent for the preparation of stock solutions of substrates and the reaction mixture usually consisted of 5 % methanol. Nevertheless, we also examined the impact of other solvents on enzyme activity, especially of water non-miscible solvents, which could give interesting results, as judged from previous works (Layh and Willetts, 1998).

This data confirmed that using methanol as a co-solvent is a good choice due to its low influence on enzyme activity at a concentration of 5%. Among water non-miscible compounds, hexane and heptane proved to be the best co-solvents. For more information, see Appendices 7 and 13.

Inorganic salts were tested with the nitrilases from *F. solani* as potential inhibitors of nitrilase activity and, not surprisingly, the strongest inhibitors were mercury and silver salts, but also zinc and aluminium salts acted as strong inhibitors of the activity, as did hydrogen peroxide.

The lyophilized mycelium proved to be the best approach for long-term storage for both *Fusarium* strains. No loss of activity was observed within a one-year period when the mycelium was stored at -20 °C (Vejvoda, 2006).

Handling the purified enzyme was more complicated. Using this preparation, continuous freezing and thawing of the enzyme should be avoided because of its deleterious effect on enzyme activity. Nevertheless, the enzyme stored at -80 °C was stable for more than half a year. For more details, see Appendices 7 and 13.

5.2.4 Immobilization

To improve enzyme stability, e.g. for long-term reaction, immobilization is generally applied. Most immobilization methods reduce the effect of co-solvents (by forming a microenvironment around the enzyme molecule), fix the structure and attenuate denaturation of the protein backbone. Importantly, immobilization also prevents the leakage of enzyme from the reaction mixture. The disadvantage of many immobilization techniques is their strong effect on enzyme structure and, hence, activity. Therefore, an appropriate technique for each enzyme must be chosen. With nitrilases, we found that mild immobilization techniques (physical adsorption) led to only a small loss of activity, but a leakage of activity was observed, and harsh immobilization techniques (covalent binding) led to significant losses of enzyme activity.

We also compared the suitability of the enzymes for use in a batch or continuous reactor. For immobilisation in a continuous reactor (stirred membrane reactor or column) permanent delivery of the substrate and removal of the product shifts the equilibrium towards the reaction products, and the leakage of activity (in case of column reactor) can be alleviated by the addition of a new portion of enzyme.

Physical sorption

This method is less invasive and takes advantage of natural enzyme properties such as the charge or hydrophobic structures on the enzyme surface. The influence on activity is probably insignificant.

Nitrilases from *A. niger* and *F. solani* O1 were adsorbed on Sepharose carriers derivatized with carbon chains of various lengths and structures and hydrophobic interaction was used for immobilization. Commercially available columns (GE Health Care Bio-Sciences AB) filled with the required carrier were used. Cell-free extracts were used for these experiments, because the economy of the process was also under consideration regarding possible application on a large scale, while purification would increase the cost of enzyme preparation.

Most tests were done with Butyl Sepharose, but Octyl and Phenyl Sepharose (all using a 1-mL volume, see Fig. 14 for illustration) were also suitable for these experiments.

It is notable that a high salt concentration (0.8 M ammonium sulfate) is needed for proper enzyme-carrier interactions, as otherwise the enzyme would be eluted from the column.

Immobilized nitrilases (*A. niger, F. solani* O1) do not show any significant differences in their pH or temperature optima compared to the purified enzymes. Both soluble and immobilized enzymes exhibited maximum activity at approximately pH 8. The maximum activities in 10-min batch experiments were determined to be in the range 40-48 °C, but over the long-term operation of the columns, the enzyme activity decreased rapidly at 45 °C. The drop of enzyme activity by one half occurred within less than 2 and 3 h for the nitrilase from *A. niger* and *F. solani,* respectively. There are probably two reasons behind this activity drop. First, it is mainly the enzyme lability, which is less pronounced for the purified enzyme than for the cell-free extract (result of batch reactions). Second, the enzyme retention on the column is not persistent, as the immobilization is only by physical sorption on the carrier surface and there is a dynamic equilibrium between the bound and mobile enzyme. This observation led us to lower the reaction temperature to 35 °C. At this temperature, the activity of the immobilized enzyme was about 70 % of its maximum activity at 45 °C, but its temperature stability increased significantly - no activity drop was observed within 2 h, and the activity did not drop below 70 % activity after 15 and 66 h with *A.niger* and *F. solani* O1, respectively (see Appendices 1, 2 and 4).

Both nitrilases were used for the continuous bioconversion of cyanopyridines with promising results. Depending on the enzyme load, the total conversion of 10 mM 4-cyanopyridine can be maintained for one day (*A. niger*, 7.2 U) or three days (*F. solani* O1, 22 U).

For removing the by-product of nitrile hydrolysis (amide) amidase was used (see Fig. 13). Two options were tested: the immobilization of nitrilase and amidase on one column (co-immobilization) and immobilization on two separate columns connected together. Both methods led to a reduction in the amount of amide, but with 4-cyanopyridine the use of columns connected in tandem reduced the amount of amide more effectively (to less than 0.2 %) and led to the production of very pure isonicotinic acid (see Fig. 14 and for more detailed information, see Appendices 2 and 4)

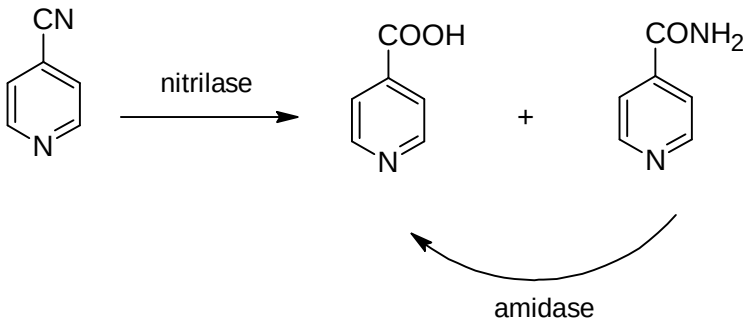


Figure 13 Two enzymes immobilization

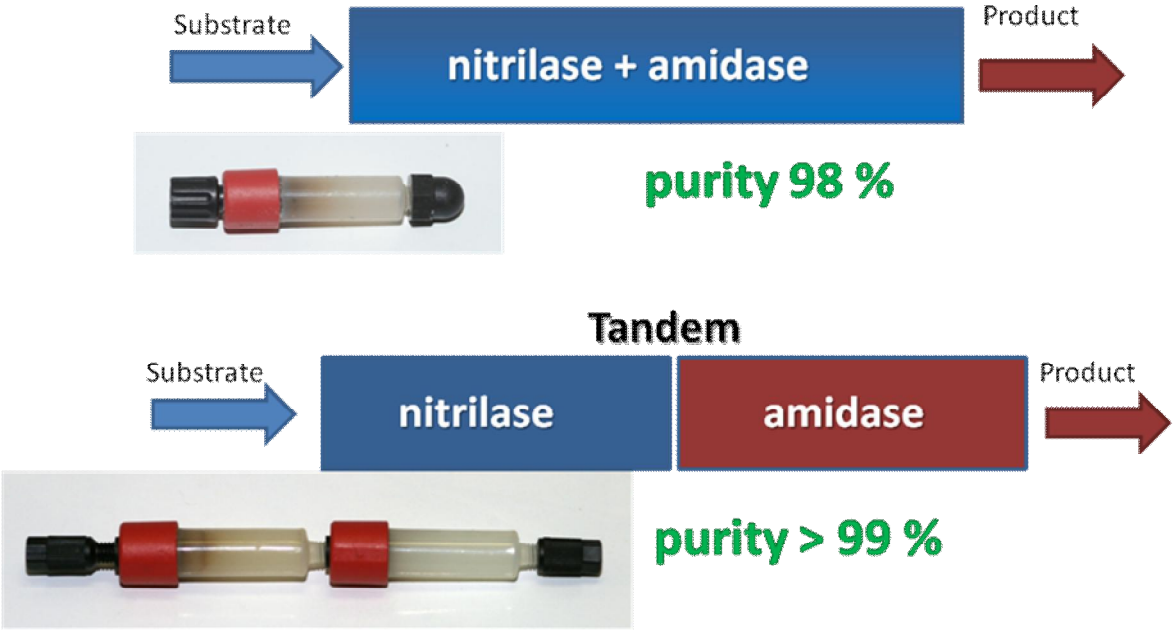


Figure 14 Patterns of two-enzyme immobilization

Continuous stirred membrane reactor (CSMR)

A CSMR was used for continuous bioconversion and for determining of thermal stability. This method is an immobilization only in the broader sense of the word, as the enzyme is held in a specific compartment by a membrane. Therefore, the enzyme is placed in the bulk solvent and the effect of the carrier on its structure is avoided.

Due to the membrane, the enzyme cannot leak from the reactor compartment, (membrane cut-off 30 kDa, subunit size 40 kDa). Thus a total loss of activity can be attributed to thermal instability, and, hence, this technique is appropriate for thermal

stability determination. It can also be used to demonstrate the enzyme's potential applicability for continuous processes and their scale-up.

Thermal stability was determined using the best substrate for all tested nitrilases, 4-cyanopyridine. The potential of enzymes for long-term, almost full bioconversion of the same substrate could also be evaluated.

The effect of various substrates on enzyme stability is different; with 4-cyanopyridine the enzymes exhibited a higher stability than with 3-cyanopyridine. Therefore, the stability is not only a function of temperature, but is multifactorial and the substrate plays an important role.

For more details see the Appendices 7 and 11.

5.2.5 Biotransformation of dinitriles

A useful application of nitrilase was demonstrated in the hydrolysis of heteroaromatic dinitriles. Dinitriles constitute a synthetically useful group of compounds because of their two functional groups, each of which can be selectively modified by chemical means or, better, by enzymes. Hence, up to 2^3 products can be obtained if all the possible combinations of nitrile, amide and carboxyl moieties are considered. Dinitrile compounds with non-equivalent nitrile groups can also be used for determining the regio-selectivity of enzymes.

From various heteroaromatic dinitriles, 2,6-pyridinedicarbonitrile and 2,4-pyridinedicarbonitrile served as the best substrates for the nitrilase from *F. solani* O1 with relative activities (benzonitrile 100 %) of 5 and 7 %, respectively. The nitrilase from *A. niger* K10 exhibited a much higher activity towards these nitriles (30 and 12 %, respectively). The other two tested substrates - 3,4-pyridinedicarbonitrile and 2,3-pyrazinedicarbonitrile - did not act as substrates for the nitrilase from *F. solani* O1, and were very poor substrates for the nitrilase from *A. niger* K10 (0.5 and 1 % of relative activity, respectively).

To obtain the desired products, the reaction conditions, especially time, were modified using a nitrilase or, alternatively, nitrile hydratase/amidase system. The results of this research are summarized in Appendix 6.

6 NITRILE HYDRATASE/AMIDASE ENZYME SYSTEM

An alternative enzymatic tool for nitrile biotransformations is the prokaryotic system of nitrile hydratase/amidase. This system consists of nitrile hydratases bearing either an Fe- or Co-type cofactor and „GGSS signature amidases“ (also designated „enantioselective amidases“), both enzymes being encoded by genes located in clusters with a number of regulatory genes. Current knowledge of the structure and function of these enzymes has been reviewed several times in recent years (Martínková et al., 2009a; Banerjee et al., 2002; Martínková and Křen, 2002; Appendix 10). The advantage of this enzyme system is its lower substrate specificity compared to nitrilases, since mainly nitrile hydratases are less sensitive to steric hindrances. The possibility of controlling the production of amides *vs.* carboxylic acids by varying the reaction conditions or reaction time is also beneficial. The significant disadvantage of nitrile hydratase, on the other hand, is its much lower stability compared to nitrilase.

This part of the work has been focused on the nitrile hydratase/amidase system of *Rhodococcus erythropoli*s A4, a typical producer of Fe-type nitrile hydratase (previously classified as *Rhodococcus equi* by biochemical tests and re-classified as *R. erythropolis* by 16S rDNA sequencing), has been used in the Laboratory of Biotransformation for a couple of years to elaborate new applications of the above enzyme in synthetic organic chemistry (for a review see Martínková and Mylerová, 2003; Kubáč et al., 2008). The nitrile hydratase from this organism has been purified and characterized (Přepechalová et al., 2001). The present work was focused on the amidase of this strain and its applications for acyl transfer reactions. The amidase has also been used in combination with nitrilase in the two-step conversion of nitrile into carboxylic acid (see above). The bienzymatic system has also been compared with fungal nitrilases, as far as its potential to transform dinitriles is concerned (see Appendix 6).

AMIDASE

Amidases have been widely applied as biocatalysts for the hydrolysis of amides. With respect to the stability of the amide bond, which requires drastic conditions for hydrolysis, a mild alternative method is very attractive. Furthermore, a large number of

amidases exhibited outstanding enantioselectivities, thus opening a straightforward route to valuable chiral carboxylic acids (Shaw et al., 2003; for reviews, see Wang, 2005; Martínková et al., 2003).

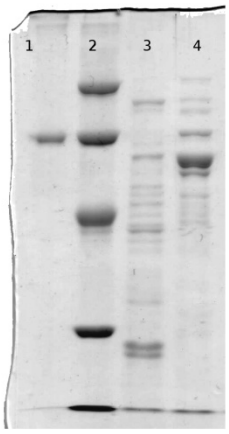
The amidases encoded by the same nitrile operon as the Fe-type nitrile hydratases in *R. erythropolis* strains (Mayaux et al., 1990; Doran et al., 2005) exhibited a high degree of amino acid identity (> 96 %), those strains being even presumably identical. The amidase in the *R. erythropolis* A4 strain differs from the these enzymes in 17 amino acid residues. Therefore, it was of interest if this enzyme may have new catalytic properties (Martínková et al., 2009b).

6.1.1 Partial purification and characterization of amidase from Rhodococcus erythropolis *A4*

For purifying the enzyme, bacterial cells grown on acetonitrile were disintegrated using an oscillation mill equipped with a stainless steel cuvette, which can be completely filled with the cell suspension leaving no space for air bubbles, thus preventing denaturation of the proteins by air. Despite these precautions, the efficiency of extraction was only about 10 % of the original activity of whole cells. This was probably caused partly by a loss of enzymatic activity, partly by incomplete extraction of the enzyme. The extract contained both amidase and nitrile hydratase activities.

The physico-chemical properties of amidase and nitrile hydratase seemed to be very similar. Therefore, it was very difficult to separate these two enzymes on Q or Butyl Sepharose columns, the main contribution of this method being the removal of ballast proteins. The separation of both only occurred in the Superdex column, which has superior resolution parameters compared to other gel filtration columns, but its disadvantage is the very low sample volume that can be applied (maximum 500 μL).

The purification of amidase was performed with a satisfactory yield of 16 %, but the increase in specific activity was less than 5-fold. This suggested that the enzyme was not purified to homogeneity, which was confirmed by SDS-PAGE (see Fig. 15). The purification protocol will need to be optimized in the future.



Lane 1 – BSA (66 kDa)

Lane 2 – Marker (20 – 97 kDa)

Lane 3 – Purified fraction after Superdex (rich in nitrile-hydratase)

Lane 4 – Purified fraction after Superdex (rich in amidase)

Figure 15 SDS-PAGE of *R. erythropolis* enzyme purification

Table 7 Purification of amidase from *R. erythropolis* A4

Step	Total protein (mg)	Specific activity (U mg^{-1})	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	10.5	1.8	19	100.0	1.0
Q Sepharose	7.0	2.1	15	80.1	1.2
Butyl Sepharose	2.5	2.1	8	41.8	1.2
Superdex 200	0.2	8.1	3	16.2	4.6

Table 8 Substrate specificity of amidase from *R. erythropolis* A4

Substrate	Relative substrate specifity (%)
Benzamide	100
Isonicotinamide	33
Nicotinamide	119
Picolinamide	36
3-Aminobenzamide	47
4-Chlorbenzamide	<1
3-Toluamide	<1
4-Toluamide	<1

The influence of co-solvents (5%) on amidase activity was similar to nitrilase. The least sensitivity was for non-water-miscible solvents (hexane, heptane) and acetonitrile - no loss of activity was observed for these solvents; a loss of approx. 5% of their activity was observed for xylene, toluene and dimethyl sulfoxide. In contrast, *n*-butanol lowered the activity to 30 % of the control. Ethanol and methanol reduced the activity to 70 % of the control. A very similar stability was also observed for the amidase from *Geobacillus pallidus* (Makhongela et al. 2007), but water non-miscible solvents were not tested for this strain with a different substrate specificity. The preferred substrates of the amidase we purified (benzamide, nicotinamide) were not subtrates for the amidase from *G. pallidus*. However, the high activities for benzamide are comparable with those of the recombinant amidase from *Microbacterium* sp AJ115 (Doran et al., 2005). The substrate specificity of the purified amidase is summarized in Table 8.

In inhibitory tests of metal ions (5 mM concentration) Hg^{2+}, Ag^{+}, Cu^{2+} and Zn^{2+} lowered the activity to less than 20 % of the control, which is comparable with the thermostable amidase from *Geobacillus pallidus* (Makhongela et al. 2007)

No or very little (less than 5%) effect was observed for Co^{2+}, Fe^{3+}, Mn^{2+}, Cr^{3+} ions (5 mM) or dithiothreitol (5 mM) reagent.

6.1.2 Acyl-transfer reaction

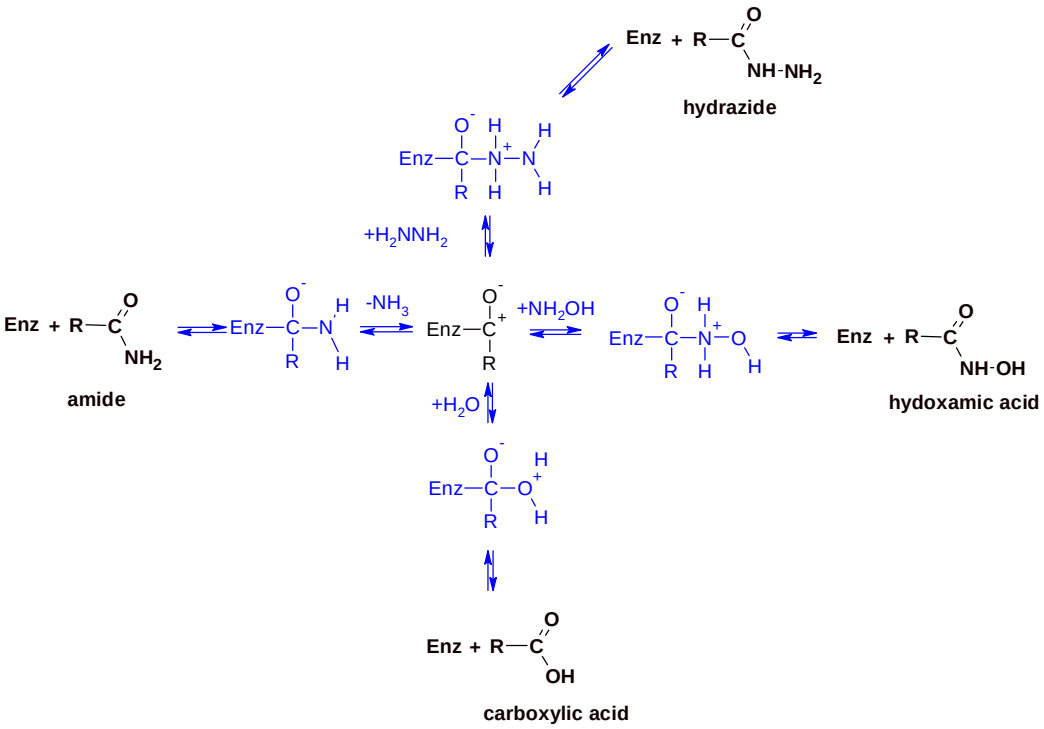


Figure 16 Amidase-catalyzed acyl-transfer reactions with hydrazine or hydroxylamine as acceptors (Fournand et al., 1998)

It has been reported that amidases are able to transfer acyls onto acyl acceptors other than water. Such acceptors can be amines but also hydroxylamine or hydrazine (Fournand et al., 1998). Reactions involving the latter two acceptors led to hydroxamates and hydrazides as valuable products (see Fig. 16).

Hydroxamates are active as chelating agents and hence find applications as antibiotics (vorinostat), growth factors or tumor inhibitors. It is not surprising that new synthetic routes towards these chemicals are of great interest. The chemical synthesis of hydroxamates is difficult and time-consuming, in contrast to the enzymatic method, which is mild, selective and fast.

We tested the amidase from *Rhodococcus erythropolis* A4 for its acyl transferase activity. The amides were prepared *in situ* from nitriles using either the nitrile hydratase from the same organism, or heterologously expressed nitrile hydratases (the latter one in

collaboration with University of Hohenheim, Institute of Food Science and Biotechnology, where these enzymes have been prepared).

Determination of hydroxamates is easy due to the chelating properties of these compounds, which produce a red-brown colour on reaction with Fe^{3+} ions, the reaction products being easily detectable by spectrophotometry at 492 nm.

Amidase and nitrile hydratase generally occur together in the same producing organism and the separation of these enzymes is (due to their similar behaviour) difficult and time consuming (see above). Therefore, the crude enzyme was used, containing both amidase and nitrile hydratase.

In the first step – nitrile to amide conversion – the nitrile hydratase activity of the crude enzyme preparation was exploited. However, the hydrolysis of the desired amide into the unwanted carboxylic acid had to be avoided. Therefore, a selective inhibitor of the amidase was needed. This task is more difficult that the impairment of nitrile hydratase, for which specific inhibitors have been found (diethylphosphoramidate; Layh and Willetts, 1998) and which is less stable than amidase. In attempting to solve this problem, we found that ammonia acted as selective inhibitor of the amidase activity (see Table 9). This finding has not yet been reported to our knowledge. The inhibitory effect is probably caused by the transfer of acyl onto ammonia instead of water as the acceptor (see Fig. 16).

Table 9 Reversible inhibition of amidase activity by NH_4^+ ions

Concentration of NH_4^+ (mM)	Relative amidase activity (%)	Concentration of NH_4^+ after dilution (mM)	Relative amidase activity (%)
720	11.5	172	14.1
480	11.5	114	22.2
320	14.4	76	19.6
160	17.3	38	30.6
80	24.5	19	48.6
40	33.1	10	59.8
0	100.0	0	100.0

Note: Reaction conditions 40 °C, pH 9, reaction time 30 min

On the other hand, ammonium ions had little effect on nitrile hydratase activity, the inhibition being observed at concentrations above 900 mM that is 450 mM ammonium sulphate (equal to 10 % of saturation).

This finding is beneficial for the selective suppression of unwanted amidase activity in crude extracts. This makes these extracts useful for amide preparation from nitriles by nitrile hydratase, while the costly purification of this enzyme may be left out.

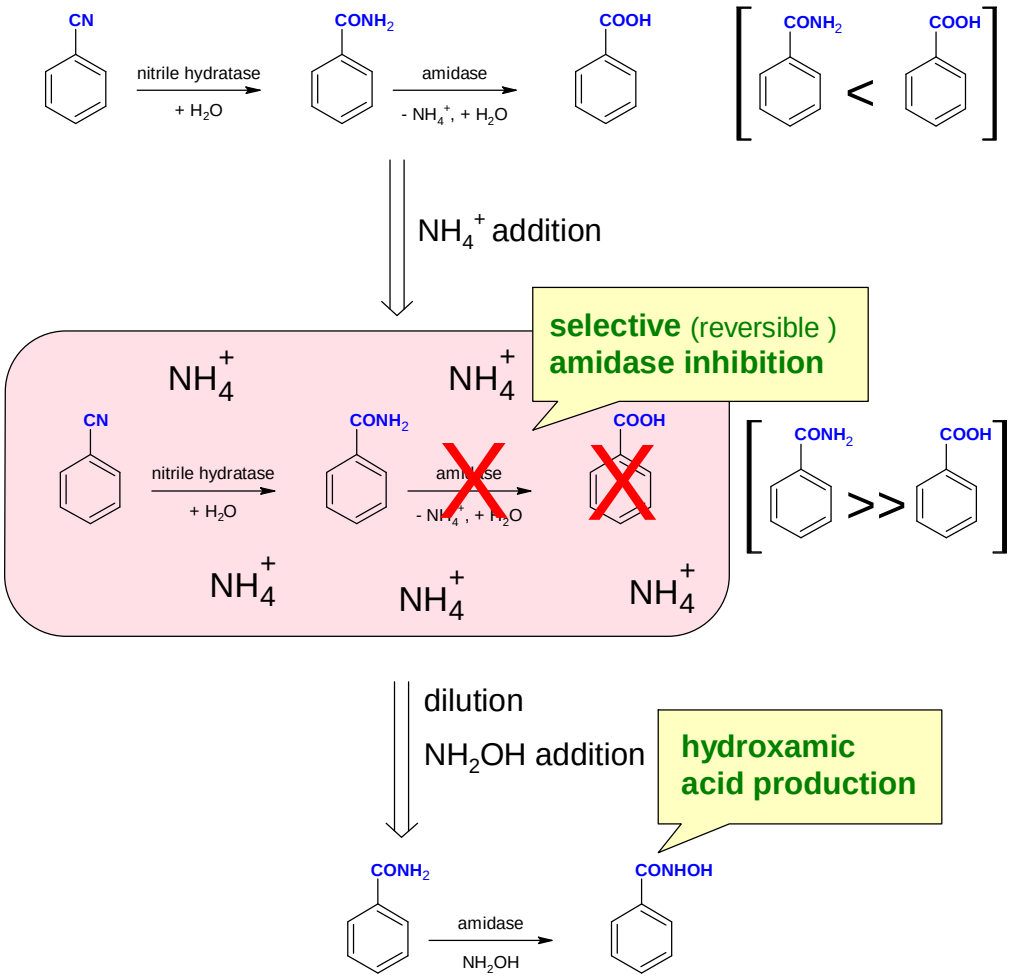


Figure 17 Hydroxamate production using amidase inhibition by ammonium ions

The two-step reaction proposed above is a promising method for the preparation of hydroxamates, as the synthesis of the starting material (nitriles) is less complicated than that of amides and, moreover, a large number of them are commercially available.

The utility of the reaction was primarily tested with benzonitrile as substrate. First we tried to use both nitrile hydratase and amidase in a one-pot reaction to produce hydroxamates directly from nitriles. However, this was impossible, because the nitrile hydratase was totally inhibited by the high concentration of hydroxylamine necessary for the acyl transfer reaction (see Table 10).

Table 10 Effect of NH_2OH on nitrile hydratase activity

NH_2OH concentration (nm)	Nitrile hydratase activity (%)
500	0.1
400	0.0
300	0.1
200	0.2
100	1.4
40	5.9
0	100.0

Note: Substrate - benzonitrile; Reaction conditions - 35 °C, pH 8, reaction time 20 min

This observation led us to perform each step of the hydroxamate synthesis separately. As an alternative to amidase inhibition by ammonia, we also used cell extracts from two heterologous producers of nitrile hydratase, which do not contain amidase activity. Therefore, loss of amide due to hydrolytic amidase activity was avoided in the first step.

Having solved the problem of amide preparation, we started to optimize the conditions of the second step - hydroxamate synthesis from amides. However, here the hydroxylamine solution also proved to suppress the reaction, especially when amidase was incubated with NH_2OH for longer periods. Nevertheless, the substrate had probably some protective influence on the amidase, as the enzyme activity decreased even more after pre-incubation with NH_2OH in the absence of substrate. A balanced concentration of hydroxylamine and substrate proved to be important. The acyl transfer reactions were performed using various amounts of benzamide (2.5 – 25 mM) and hydroxylamine (50 – 500 mM) and the optimum concentrations were found to be 500 mM of hydroxylamine and 15 mM of substrate.

Using this method, we found that the amidase from *R. erythropolis* A4 is able to produce hydroxamic acids from a number of amides. The substrate specificity of the amidase in acyl transfer reactions is shown in Table 11 and substrate specifities of recombinant nitrile hydratases is summarized in Table 12.

Table 11 Substrate specificity of amidase from *Rhodococcus erythropolis* A4 for acyl transferase activity

Substrate	Response level with amidase	Response of spontaneous reaction
Benzamide	+++	-
3-Toluamide	+++	-
4-Toluamide	++	-
2-Chlorobenzamide	++	-
4-Chlorobenzamide	+	-
3-Aminobenzamide	++	-
4-Aminobenzamide	+	-
Nicotinamide	-	-
Isonicotinamide	-	-
Propionamide	+++	-
Butyramide	+++	-
Valeramide	+++	-
Cyclohexancarboxamide	+++	-
Methacrylamide	++	+
L-Phenylglycinamide	-	-
D-Phenylglycinamide	-	-
D,L-Lactamide	++	++

– no or very poor response

Note: Amides (15 mM each) were incubated with the cell-free extract from *R. erythropolis* A4 and hydroxylamine (500mM) in Tris/HCl buffer (50 mM, pH 8) at 28 °C for 200 min. Amidase was omitted from the blanks. Response was determined spectrofotometrically at 492 nm.

Table 12 Substrate specificity of recombinant nitrile hydratases determined spectrophotometrically after coupling with enzymatic acyl transfer

Substrate	Response level with nitrile hydratase from		Response of spontaneous non-enzymatic reaction
	strain 38.1.2.	strain 77.1.	
Benzonitrile	++	+	-
3-Tolunitrile	++	+	-
4-Tolunitrile	+	+	+
3-Chlorobenzonitrile	+	-	-
4-Chlorobenzonitrile	+	+	-
3-Hydroxybenzonitrile	+	+++	-
4-Aminobenzonitrile	+	+	-
Propionitrile	++	++	+
Butyronitrile	+++	++	+
Valeronitrile	+++	++	+
2-Methyl-3-butenenitrile	+	+	+

Note: The nitriles (15 mM each) were incubated with a recombinant nitrile hydratase at room temperature for 2 days. Afterwards, hydroxylamine (500mM) and an appropriate amount of the cell-free extract from *R. erythropolis* A4 were added and the acyl transfer reaction performed in Tris/HCl buffer (50 mM, pH 8) for 200 min at 28 °C. Amidase was omitted from the blanks. Response was determined spectrofotometrically at 492 nm.

7 DEVELOPMENT OF SCREENING AND ASSAY METHODS

Most of the analytical methods in the field of nitrile-degrading enzymes are based on compound separation by HPLC, which is usually laborious and time consuming. Only one tenth of the methods used are faster spectral methods and most of them are NMR or IR, requiring significant expertise and expensive devices. Fast, simple and robust analytical methods such as those based on UV/VIS spectrometry are important for activity determination, especially when very high numbers of samples are to be analyzed as, e.g., in the screening of mutant strains. The analytical methods suitable for nitrilase assays and screening are overviewed in Appendix 8. We focused our work on screening methods based on the change in pH during the nitrilase-catalyzed reaction and on the differing UV spectra of substrates and products. Nevertheless, we also paid attention to the optimization and acceleration of HPLC methods.

CHROMATOGRAPHY

In most cases, HPLC was used to monitor the concentrations of nitriles and their reaction products and to calculate enzyme activity.

As all the non-aliphatic substrates examined here have a dominant hydrophobic moiety, a silica gel matrix derivatized with C18 or C8 carbon chains was used as the stationary phase. The mobile phase contained various concentrations of acetonitrile as the commonly used solvent.

Conventional chromatography columns filled with a particle stationary phase were replaced with monolithic columns during our work. The monolithic columns allowed us to decrease the run time of analyses and, in this way, to decrease the amount of organic solvent consumed despite the higher flow rates (usuallly a saving of 70 % of organic solvent compared to particle column analysis). At the same time, a satisfactory resolution was observed for all determined compounds. With benzonitrile (the reference substrate), the analysis time was shortened from 10 minutes (with particle-filled columns) to less than 2 minutes (see Appendix 8). These results are not the limit of the column - by increasing the flow rate, the analysis may be shortened even further if using another detector suitable for detecting peaks in analyses lasting less than one minute.

A comparison of both methods is shown in Table 13, demonstrating retention times.

Table 13 Comparison of retention times of selected analytes on particle and monolithic column

Analytes	Particle column	Monolithic column	Mobile phase acetonitrile (%) particle/monolithic
	RT (min)		
4-Aminobenzoic acid	2.1	0.51	17 / 10
2-Hydroxybenzonitrile	7.8	1.97	17 / 10
3-Hydroxybenzonitrile	7.7	1.82	17 / 10
3-Hydroxybenzoic acid	3.6	1.09	17 / 10
4-Hydroxybenzonitrile	6.3	1.52	17 / 10
4-Hydroxybenzoic acid	2.8	0.80	17 / 10
2-Tolunitrile	10.7	2.29	30 / 20
3-Tolunitrile	12.1	2.59	30 / 20
3-Toluic acid	5.4	1.52	30 / 20
4-Tolunitrile	11.3	2.44	30 / 20
4-Toluic acid	5.2	1.48	30 / 20
2-Chlorobenzonitrile	10.0	2.34	30 / 20
3-Chlorobenzonitrile	13.1	2.73	30 / 20
3-Chlorobenzoic acid	6.7	2.56	30 / 20
Benzonitrile	5.7	1.74	30 / 20
Benzoic acid	3.3	1.18	30 / 20
Benzamide	1.9	0.87	30 / 20

Note: Particle column – NovaPak RP-18 (Waters), 150mm×3.9mm i.d., 4 μm;
Monolothic column - Chromolith Flash RP-18 (Merck),25mm×4.6mm i.d.

Detection with a DAD detector was sufficient despite its lower sensitivity in comparison with a single-wavelength detector. On the other hand, the great advantage of a DAD detector was its ability to identify reaction products not only by their retention times, but also by their UV spectrum. This facilitates reading the chromatograms of complex reaction mixtures, where several product peaks occur, such as, e.g., in the bioconversion of dinitriles where more than four products may arise from a single substrate.

Modern columns, especially the monolithic columns tested in our laboratory with excellent results, mean a significant improvement in HPLC analysis, not only in terms of savings in mobile phase consumption, but primarily in instrument time. For detailed information, see Appendices 7 and 8.

SPECTROPHOTOMETRY

7.2.1 pH- Responsive method

For this method benzonitrile, 3-chlorobenzonitrile, 3- and 4-cyanopyridine and acetonitrile were used as substrates. All the corresponding acids have a pK_a in the range between 3.82 and 4.96. The reaction mixture consisted of a buffer, pH indicator, enzyme preparation, substrate and methanol as co-solvent. The buffers and pH indicators used are summarized in Table 14. The substrates themselves did not influence the colour change in blank reactions.

The best catalyst for these reactions was the lyophilized mycelium, followed by the cell-free extract of this mycelium buffered to a given pH. Nevertheless, the pH of the extract influenced the pH changes in the reaction mixture especially at alkaline pH, and using blank samples is very important in this case.

At pH $\geq$ 9, the buffer concentration had to be higher than 10 mM, because a low buffering capacity would cause changes in pH after the addition of the enzyme preparation. For pH $\leq$ 8, a buffer concentration of 10 mM seems to be satisfactory. For pH between 7.6 and 8, bromothymol blue, neutral red and phenol red are the pH indicators of choice and the colour change is significant. At pH 9.6 using phenolphtalein and thymol blue is satisfactory, but these pH indicators are too sensitive and a small change in the reaction mixture composition will cause a colour change. Ammonia formed during nitrilase reactions did not cause any significant colour change. None of the tested indicators (up to 10 % of reaction mixture volume) caused any inhibition of the reaction (tested by HPLC).

Table 14 Wavelengths at which maximum change in absorbance of the reaction mixture and blank was observed during nitrilase-catalyzed reaction in the presence of various pH indicators

Indicator	Colour change (pH)	$\Delta\lambda_{max}$ pH 7.2 (nm)	$\Delta\lambda_{max}$ pH 8 (nm)	$\Delta\lambda_{max}$ pH 9.6 (nm)
Bromothymol blue	6 - 7.6	442	617	616
p-Nitrophenol	5.6 – 7.6	-	-	-
Neutral red	6.8 - 8	535	534	532
Phenol red	6.8 – 8.4	428	426	559
Cresol red	7.2 – 8.8	426	428	573
Cresol purple	7.4 - 9	413	426	426
Litmus	5 -8	586	588	589
Phenophtalein	8.3 - 10	-	-	555
Thymol blue	8 – 9.6	-	-	596
mixed indicators				
Neutral red : bromothymol blue (1:1)	7.2	536	534	533
Bromothymol blue : phenol red (1:1)	7.5	436	560	560
Cresol red : thymol blue (1:3)	8.3	437	431	574

Table 15 Buffers used for nitrilase assays based on monitoring pH change

Buffer	Concentration (mM)	pH
TRIS/HCl	10 - 50	7.2 - 9.6
Na/K phosphate buffer	10	7.2
Triethanol amine/EDTA/NaOH	10	7.2
$NaHCO_3$/ Na_2CO_3	10 - 50	9.6
Glycine/NaOH	10	9.6

Table 16 Usability of pH indicators at different pH of nitrilase-catalyzed reactions (0 – unusable, ++ - very useful).

Indicator	pH 7.2	pH 8	pH 9.6
Bromothymol blue	++	++	0
p-Nitrophenol	0	0	0
Neutral red	++	++	0
Phenol red	+	++	0
Cresol red	0	+	+
Cresol purple	0	0	+
Litmus	+	+	0
Phenophtalein	0	0	++
Thymol blue	0	0	++
mixed indicators			
Neutral red : bromothymol blue (1:1)	+	++	0
Bromothymol blue : phenol red (1:1)	+	++	0
Cresol red : thymol blue (1:3)	0	0	+

7.2.2 UV-Spectrometric measurements

UV methods have been rarely used for direct measurement of the substrates and products of nitrilase-catalyzed reactions so far. This was caused by some difficulties connected with this type of measurement. Namely, cyano, carboxamido or carboxy groups exhibited a low absorbance in the UV/VIS range, and in most cases only small differences between the substrate and product were observed. The reason for this is that the main contribution to absorbance originates from the carbon chain. It is obvious that only aromatic compounds or compounds with conjugated double bonds are suitable for UV measurements.

The preliminary experiments were carried out in cuvettes using a double beam UV spectrophotometer, but this method was too time-consuming. However, one of its advantages was the possibility of adapting the method for a microplate reader. Only special plates with a bottom made of a plastic foil transparent in the near-UV could be used for this purpose. Magellan software facilitated the work-up and interpretation of the data.

In total, 16 substrates were tested using this method and, surprisingly, it was found that nearly all tested compounds were suitable for this method, although the difference between the maximum absorption of the substrate and product was less than 5 nm. The difference between the spectrum of the substrate and product is illustrated in Fig. 18.

By using standards at concentrations of less than 1 mM, we were able to determine the absorption coefficients of the compounds and to find the wavelength at which the difference in spectrum of substrate and product was maximal.

It is possible to distinguish between the substrate and product via this method, using not only the difference in their absorption maxima, but also the difference in their absorption coefficients at a certain wavelength. The results obtained for selected compounds are summarized in

Table 17.

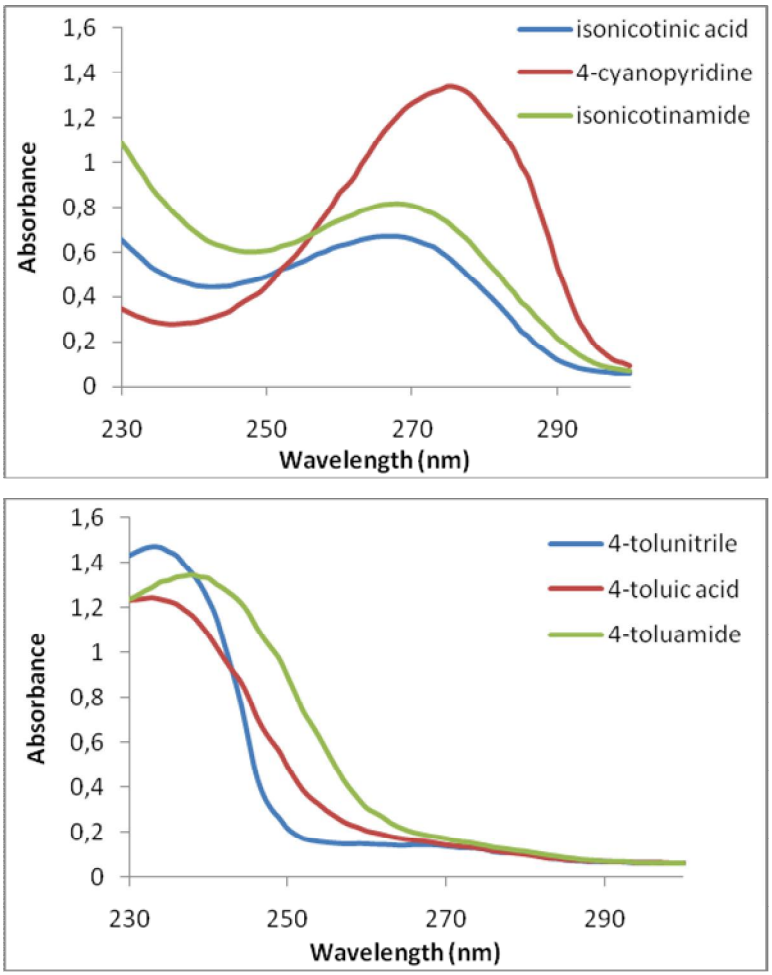


Figure 18 Examples of spectra of substrate and products

A benefit of this method in comparison to the end-point method (e.g. HPLC) is that the reaction can be monitored continuously and, therefore, inhibition or activation effects can be monitored, and also kinetic parameters can be easily calculated.

However, the possibility of the formation of other compounds such as amide may complicate the evaluation and makes this method only suitable for semi-quantitative measurements. Therefore, other methods must be used if a detailed analysis of the reaction mixture components is desired. On the other hand, the UV method is very fast and therefore useful for screening (analysis of fractions during purification, screening for active mutant enzymes etc., see Appendix 7).

Table 17 Selected substrates and possible products of their transformation by nitrilase (corresponding acid and amide)

Wavelength (nm)	Product			Substrate			Difference in molar absorptivity ($cm^2.mol^{-1}$)	Ratio of molar absorptivity
	Product of bioconversion	Molar absorptivity ($cm^2.mol^{-1}$)	Absorption maximum (nm)	Substrate of bioconversion	Molar absorptivity ($cm^2.mol^{-1}$)	Absorption maximum (nm)		
264	nicotinic acid	3.0 ± 0.1	230 (263)	3-cyanoypridine	7.4 ± 1.4	265 (230)	-4.3	0.411
230	nicotinic acid	2.6 ± 0.3	230 (263)	nicotinamide	5.2 ± 0.2	230 (262)	-2.6	0.504
264	nicotinamide	2.6 ± 0.1	230 (262)	3-cyanoypridine	7.4 ± 1.4	265 (230)	-4.8	0.353
278	isonicotinic acid	1.8 ± 0.2	230 (266)	4-cyanopyridine	3.8 ± 0.7	275	-2.0	0.477
230	isonicotinic acid	1.2 ± 0.3	230 (266)	isonicotinamide	3.0 ± 0.2	230 (267)	-1.8	0.393
278	isonicotinamide	2.1 ± 0.1	230 (267)	4-cyanopyridine	3.8 ± 0.7	275	-1.7	0.560
238	benzoic acid	4.3 ± 0.2	230	benzamide	6.7 ± 0.4	230	-2.4	0.645
238	benzoic acid	4.3 ± 0.2	230	benzonitrile	1.2 ± 0.1	231	3.1	3.655
238	benzamide	6.7 ± 0.4	230	benzonitrile	1.2 ± 0.1	231	5.5	5.671
247	4-toluic acid	5.9 ± 0.3	233	4-toluamide	10.4 ± 0.3	235-238	-4.6	0.562
247	4-toluic acid	5.9 ± 0.3	233	4-tolunitrile	2.4 ± 0.1	232-234	3.5	2.457
247	4-toluamide	10.4 ± 0.3	235-238	4-tolunitrile	2.4 ± 0.1	232-234	8.0	4.375
231 / 252	4-chlorobenzoic acid	11.2 ± 0.6 / 3.2 ± 0.1	232-235	4-chlorobenzamide	12.5 ± 0,8 / 7.3 ± 0.4	237-244	-1.3 / -4.1	0.898 / 0.438
278	4-hydroxybenzoic acid	2.3 ± 0.2	246	4-hydroxybenzonitrile	12.7 ± 0.6	273	-10.4	0.181
230 / 285	3,5-dibromo-4-hydroxybenzoic acid	15.3 ± 1.2 / 13.3 ± 0.9	230 (283)	bromoxynil	19.1 ± 1.9 / 16.9 ± 0.8	230 (283)	-3.8 / -3.6	0.802 / 0.788
259 / 284	3,5-diiodo-4-hydroxybenzoic acid	6.0 ± 0.4 / 14.1 ± 0.7	235 (284)	ioxynil	5.5 ± 0.5 / 16.4 ± 0.7	236 (284)	0.6/ -2.3	1.103 / 0.862

8 CONCLUSION

The trend in enzymology is from finding new enzymes *in vivo* to preparing enzymes *in vitro* by altering natural proteins or by the synthesis of completely new ones. However, without broad knowledge and a good understanding of natural enzymes, this new trend cannot be efficient.

Nitrilases have been known about since the 1960s, but, as stated above, not all features of these enzymes are understood. In some cases we still have to say *hic sunt leones*.

In this work, we tried to describe new enzymes and obtain information about their structure and function.

Both described *Fusarium* strains are originally soil isolates with minor nitrilase activity levels (less than 4 $U.L^{-1}_{medium}$), but changing the inducer and cultivation procedure to a two-step cultivation led to an enhancement of their activity to thousands of units per litre of medium. Moreover, lyophilization of the mycelium obtained in this way stabilizes the enzyme for more than a year, which makes the enzyme accessibility very simple and straightforward.

New nitrilases purified and characterized in this work seem to belong to enzymes preferentially accepting aromatic nitriles, and to be not too different in terms of reaction conditions from other, previously described nitrilases. They are not so coherent in their structure, because some bacterial nitrilases are less than 200 kDa in molecular weight (Thuku et al., 2009), but the nitrilases purified by us seem to be more than 440 kDa. Electron microscopy is the tool of choice in revealing their quaternary structure. This technique enabled observation of the enzymes maturing by assembling into helical rods (Thuku et al., 2007) with a molecular weight exceeding the upper molecular weight limit of conventional columns. The size of these nitrilase species makes precise determination of the molecular weight of nitrilases difficult. The crystallography analysis of nitrilases will be very useful as the crystal structure has not been determined yet for any of these enzymes, and it is the aim of our group in future research (Kaplan et al., 2010).

To determine the applicability of enzymes for the preparation of carboxylic acids, different immobilization techniques were tested in this work. The continuous stirred

membrane reactor (CSMR) was found to be a useful technique, not only for the preparation of products but also for studying enzyme stability and behaviour in continuous mode. The disadvantage of this laboratory-scale CSMR used here is its low productivity (due to a low flow rate), but its advantage is a low consumption of enzyme. Nevertheless, all tested immobilization techniques improve enzyme stability and are suitable for the possible preparation of interesting enzymatic reaction products.

Another enzymatic system belonging to the nitrilase superfamily is the nitrile hydratase/amidase system from the bacterium *Rhodococcus erythtropolis* A4. A specific reversible inhibitor (ammonium ions) was found for the amidase, making the use of the crude extract more profitable and time-saving (alternative for enzyme purification). The application of an amidase in the preparation of hydroxamic acids makes this enzyme very promising, because of the high value of hydroxamic acids in pharmaceuticals and elsewhere.

This work has broadened the portfolio of nitrile metabolism enzymes a little and new features of the enzymes were determined. At the same time, a new method of fast activity determination was elaborated.

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4) 24. kongres Československé společnosti mikrobiologické, Liberec 2. – 5. 10. 2007
5) Biocat, 4th international congress on biocatalysis, 31. 8. – 4. 9. 2008, Hamburg
6) Cost, CASCAT, Kick-off meeting, 18. – 20. 9. 2008, Como, Italy

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Short stay abroad

Germany (27.5.2007 – 23. 6. 2007) Stuttgart, University of Hohenheim, Institute of Food Science and Biotechnology, supported by DAAD-PPP Project.

11 APPENDICES

Related published articles

Appendix 1

Vejvoda V., Kaplan O., Bezouška K., Martínková L.: Mild hydrolysis of nitriles by the immobilized nitrilase from *Aspergillus niger* K10. *Journal of Molecular Catalalysis B: Enzymatic,* 2006: 39, 55-58.

Appendix 2

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Appendix 3

Kaplan O., Vejvoda V., Charvátová-Pišvejcová A., Martínková L.: Hyperinduction of nitrilases in filamentous fungi. *Journal of Industrial Microbiology and Biotechnology*, 2006: 33, 891-896.

Appendix 4

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Appendix 5

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