CHARLES UNIVERSITY IN PRAGUE

Faculty of Science

Department of Biochemistry

Study program: Biochemistry

Field of study: Biochemistry

Dissertation

Structure and biocatalytic properties of nitrilases from filamentous fungi

Mgr. Alena Petříčková

Supervisor: Ing. Ludmila Martínková, CSc.

Prague 2013

KARLOVA UNIVERZITA V PRAZE

Přírodovědecká fakulta

Katedra biochemie

Studijní program: Biochemie

Studijní obor: Biochemie

Disertační práce

Struktura a biokatalytické vlastnosti nitrilas z vláknitých hub

Mgr. Alena Petříčková

Školitel: Ing. Ludmila Martínková, CSc.

Praha 2013

I hereby declare that this thesis is based on my own research carried out in the Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., except where due acknowledgement has been made in the text, and that all sources of information are cited. No part of the work was used for obtaining the same or different academic title.

In Prague Signature

Acknowledgements

I would like to thank all of my colleagues from the Laboratory of Biotransformation (Institute of Microbiology, Academy of Sciences of the Czech Republic), especially my supervisor Ing. Ludmila Martínková, CSc. for her help, scientific advice and leadership of my dissertation, and the head of laboratory, Prof. Ing. Vladimír Křen, DrSc., for his support.

I would also like to thank all of my co-workers, RNDr. Oldřich Benada, CSc. (Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences of the Czech Republic) for the electron microscopy analysis, Ing. Natalia Kulik, PhD. (Institute of Nanobiology and Structural Biology, Academy of Sciences of the Czech Republic) for the homology modelling, Mgr. Ondřej Vaněk, PhD. (Department of Biochemistry, Faculty of Science, Charles University in Prague) for the sedimentation velocity analysis, Ao.Univ.-Prof. Dr. Norbert Klempier, PhD. and Dipl.-Ing. Birgit Wilding (Institute of Organic Chemistry, Graz University of Technology) for providing a nitrilase substrate and valuable advice.

Special thanks belong to apl. Prof. Dr. Andreas Stolz and his work group from the Institute of Microbiology, University of Stuttgart, for their patient help during my three-month stay in Germany.

Finally, I would like to thank my partner and family for their patience and support.

The work was supported through the following grants:

P504/11/034 (Czech Science Foundation), IAA500200708 (Grant Agency of the Academy of Sciences of the Czech Republic), TA01021368 (Technology Agency of the Czech Republic), European Cooperation in Science and Technology (COST) Action CM0701 (short-term scientific mission fellowship COST-STSM-CM0701-6753), OC09046 (Ministry of Education of the Czech Republic)

Abstract

Filamentous fungi are alongside bacteria a rich source of nitrilases that can be used for biotechnological purposes. There is a large number of sequences of putative nitrilases in databases, but few of these enzymes have been expressed, purified and characterized.

According to the sequences in GenBank database, synthetic genes of putative nitrilases from *Aspergillus niger* CBS 513.88 and *Neurospora crassa* OR74A were designed, expressed in *Escherichia coli* and the nitrilases produced were purified and characterized. The effect of chaperone-assisted nitrilase expression was also examined using a set of commercial chaperone plasmids.

The structural properties of nitrilases were examined using various methods, such as homology modelling, sedimentation velocity analysis or electron microscopy.

Recombinant nitrilase production was compared to their production by native producers – filamentous fungi. The cultivation of native producers was rather difficult and nitrilase production was very low, therefore, the advantage of using prokaryotic expression system was highlighted.

Nitrilases with amino acids close to the catalytic cysteine replaced were prepared in order to study the effects of certain amino acids on the activity, chemoselectivity and enantioselectivity of nitrilases. The catalytic properties of putative nitrilases found in databases can be partially predicted, due to the similar effects of certain amino acids close to the catalytic centre of the nitrilase on enantioselectivity and chemoselectivity in distantly related nitrilases.

Both aforementioned nitrilases were able to convert a high concentration of (*R,S*)-mandelonitrile up to 500 mM with high enantioselectivity for the formation of (*R*)-mandelic acid. Therefore, a potential application was found for these nitrilases in the synthesis of optically pure carboxylic acids or amides from racemic substrates in high concentrations.

Abstrakt

Vláknité houby jsou vedle bakterií bohatým zdrojem nitrilas využitelných pro biotechnologické účely. V databázích lze najít velké množství sekvencí domnělých nitrilas, ale jen málo těchto enzymů bylo exprimováno, purifikováno a charakterizováno.

Syntetické geny nitrilas z *Aspergillus niger* CBS 513.88 a *Neurospora crassa* OR74A byly navrženy podle sekvencí uložených v databázi GenBank. Tyto geny byly exprimovány v *Escherichia coli* a získané nitrilasy byly purifikovány a charakterizovány. Také byla studována ko-exprese molekulárních chaperonů s nitrilasami pomocí sady komerčních chaperonových plasmidů.

Pro studium strukturních vlastností nitrilas byly použity různé metody, jako je homologní modelování, analytická ultracentrifugace nebo elektronová mikroskopie.

Produkce rekombinantních nitrilas byla srovnána s produkcí nativními producenty – vláknitými houbami. Vzhledem k nízké produkci enzymu těmito houbami a jejich obtížné kultivaci se ukázalo být výhodným využití prokaryotického expresního systému.

Vliv aminokyselin v blízkosti katalytického cysteinu na aktivitu, chemoselektivitu a enantioselektivitu byl studován pomocí bodových mutací. Vliv určitých aminokyselin v blízkosti katalytického centra enzymu na jeho vlastnosti je podobný i u vzdáleně příbuzných nitrilas, proto je možné katalytické vlastnosti domnělých nitrilas v databázích částečně předpovědět.

Obě zmíněné nitrilasy byly schopné přeměnit (*R,S*)-mandelonitril o koncentraci až 500 mM na (*R*)-mandlovou kyselinu s vysokou enantioselektivitou. Tyto nitrilasy tak mohou být využity pro syntézu opticky čistých karboxylových kyselin nebo amidů z racemických směsí nitrilů o vysokých koncentracích.

List of content

Abbreviations

Ala	alanine
ATP	adenosintriposphate
BLAST	basic local alignment search tool
CLEAs	cross-linked enzyme aggregates
Cys	cysteine
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
e.e.	enantiomeric excess
Glu	glutamic acid
IAN	3-indolylacetonitrile
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria – Bertani
Lys	lysine
MA	mandelic acid
MAA	mandelamide
MN	mandelonitrile
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NHase	nitrile hydratase
PDA	photodiode array
PPA	2-phenylpropionic acid
PPAA	2-phenylpropionamide
PPN	2-phenylpropionitrile
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan
UDPG-glucosyltransferase	uridine diphosphoglucose-glucosyltransferase

1 Introduction

1.1 Nitriles and cyanides in nature

Nitriles are generally toxic compounds due to the presence of a cyano group in their molecules. Moreover, some of them are mutagenic and carcinogenic. Cyanides are fast-acting poisons, which block electron transport during the respiratory cycle by binding the iron ion of cytochrome c oxidase. In this way, the synthesis of ATP is inhibited (Gupta *et al.*, 2010).

Most nitrile compounds in nature originate from plants. They occur in various forms such as cyanogenic glycosides, cyanolipids or various simple nitriles (3-phenylpropionitrile (**1**), phenylacetonitrile (**2**), 3-indolylacetonitrile (**3**) and ricinine (**5**)), etc. (Figure 1.1) (Banerjee *et al.*, 2002).

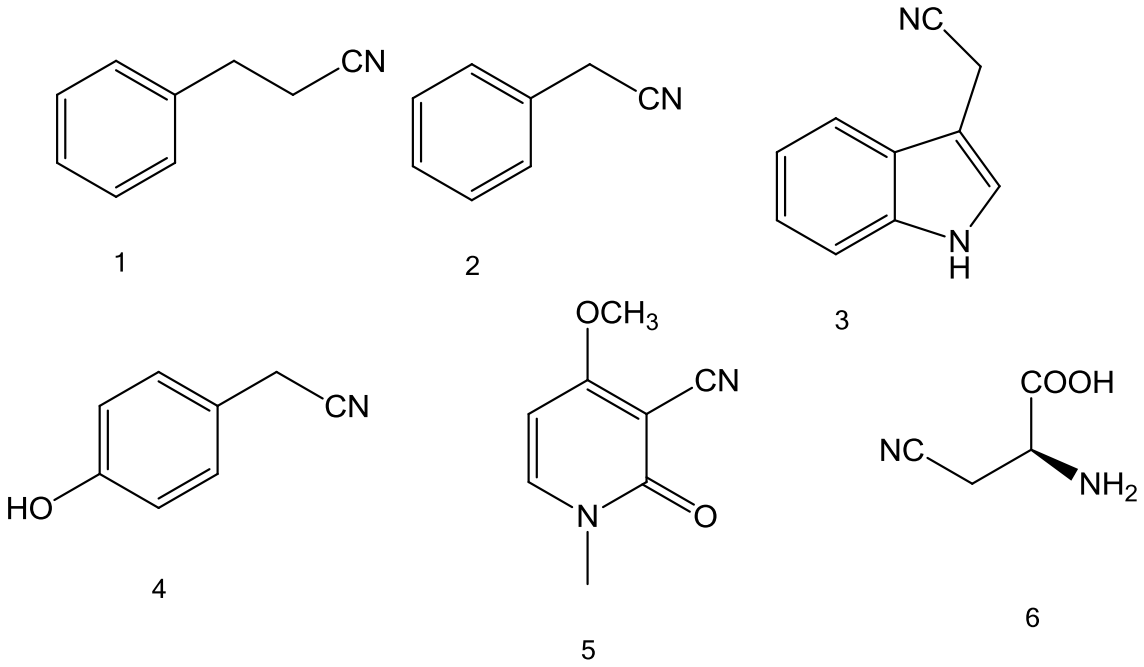


Figure 1.1: Examples of nitriles occurring in nature: 3-phenylpropionitrile (1), phenylacetonitrile (2), 3-indolylacetonitrile (3), 4-hydroxyphenylacetonitrile (4), ricinine (5), β-cyano-L-alanine (6)

Cyanogenic glycosides are the largest group of natural nitrile compounds. They are defined as β-glycosides of α-hydroxynitriles and derive from amino acids (valine,

isoleucine, leucine, phenylalanine, tyrosine or cyclopentyl glycine); the sugar moiety is D-glucose in most cases. Cyanogenic glycosides are found in more than 2500 plant species (Vetter, 2000). Some typical cyanogenic glycosides are linamarin (**7**), lotaustralin, linustatin (derived from valine and isoleucine); cardiospermin (derived from leucine); prunassin, (*R*)-amygdalin (**9**) (derived from phenylalanine); dhurrin (**8**) and taxyphyllin (derived from tyrosine) (Figure 1.2) (Legras *et al.*, 1990).

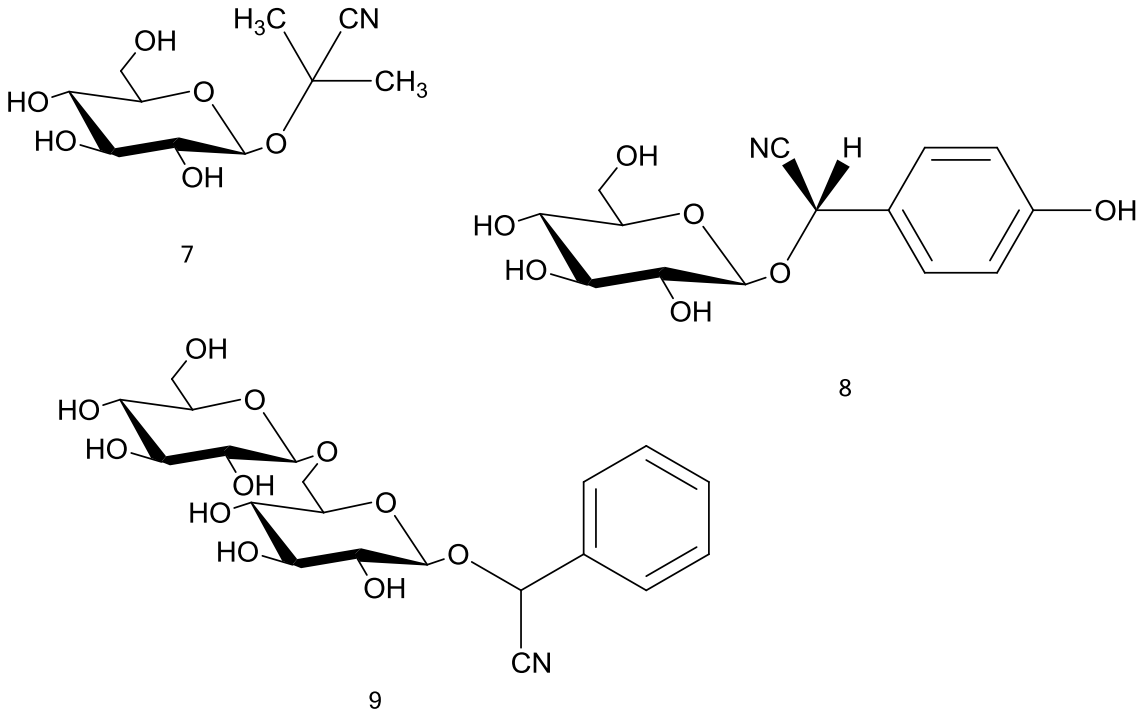


Figure 1.2: Examples of cyanogenic glycosides: linamarin (7), dhurrin (8), amygdalin (9)

Upon tissue disruption, β-glucosidases (which are normally physically separated from the substrate in plant cells) and α-hydroxynitrile lyases hydrolyze the cyanogenic glycosides releasing hydrogen cyanide and a ketone or aldehyde (Zagrobelny *et al.*, 2004). Therefore, these compounds, along with glucosinolates, may play a role in phytopathogenic resistance. Glucosinolates have a structure comprised of a β-D-thioglucose group linked to a sulfonated aldoxime moiety and a variable side chain derived from amino acids. Upon tissue damage, they are decomposed by myrosinases (EC 3.2.1.147; systematic name thioglucoside glucohydrolase) into unstable aglycons, which are typically further rearranged typically into nitriles or isothiocyanates (Wittstock and Halkier, 2002).

Cyanolipids are fatty acid esters of certain hydroxynitriles derived from leucine. Cyanolipids are classified into four groups according to the structure of the α-hydroxylated nitrile (Legras *et al.*, 1990). Their function might be the storage and transport of reduced nitrogen (Bjarnholt and Møller, 2008).

3-Indolylacetonitrile (IAN) is a precursor of a major plant hormone – 3-indolylacetic acid. IAN can be formed in *Brassicaceae* from glucosinolate glucobrassicin and further metabolized by nitrilase (Piotrowski, 2008).

4-Hydroxyphenylacetonitrile (**4**) is an intermediate in the biosynthesis of dhurrin. Upon tissue disruption 4-hydroxymandelonitrile is released, which is converted to 4-hydroxybenzaldehyde and hydrogen cyanide. Dhurrin can also be metabolized to 4-hydroxyphenylacetonitrile, which is further converted by nitrilase to the corresponding acid and ammonia (Piotrowski, 2008).

β-Cyano-L-alanine (**6**) is produced in plants from hydrogen cyanide and cysteine or serine in a cyanide detoxification pathway. β-Cyano-L-alanine is further converted to a mixture of asparagine, aspartic acid and ammonia in a reaction catalyzed by β-cyano-L-alanine nitrilase (Miller and Conn, 1980).

Hydrogen cyanide is formed in plants during the decomposition of some natural nitriles - cyanohydrins, cyanogenic glycosides and cyanolipids. Hydrogen cyanide is also formed as a by-product in the biosynthesis of the plant auxin ethylene (Legras *et al.*, 1990).

The best explored nitrilase-producing plant is *Arabidopsis thaliana* (belonging to *Brassicaceae*). This plant expresses four types of nitrilase, NIT1, NIT2, NIT3 and NIT4. NIT1 to NIT3 and their homologues are not active on β-cyano-L-alanine, but can use a broad spectrum of aliphatic and aromatic nitriles as substrates – 3-indolylacetonitrile, 4-hydroxyphenylacetonitrile, 3-phenylpropionitrile, etc. (Osswald *et al.*, 2002). NIT1 to NIT3 are similar to each other with 84-90 % amino acid identity and less similar to NIT4 with 66-68 % amino acid identity. NIT1 to NIT3 homologues are probably responsible for the degradation of nitriles from glucosinolates (defence compounds) in *Brassicaceae* (Vorwerk *et al.*, 2001; Janowitz *et al.*, 2009).

NIT4 and its homologues (β-cyano-L-alanine nitrilases) are specific for β-cyano-L-alanine and can be probably found in all plants, because β-cyano-L-alanine is

widespread in the plant kingdom as a product of hydrogen cyanide detoxification (Osswald *et al.*, 2002).

Not only plants but also bacteria, fungi and animals produce nitrile compounds. The presence of nitrile compounds in animals is limited, nitriles were only found in sponges and arthropods, where they exhibit antimicrobial activities and provide protection against predators (Legras *et al.*, 1990).

1.1.1 Biosynthesis of nitrile compounds

The biosynthesis of cyanogenic glycosides – a major group of natural nitrile compounds – is catalyzed by two multifunctional cytochromes P450, and UDPG-glucosyltransferase (Bjarnholdt and Møller, 2008). The aglycones of cyanogenic glycosides are derived from amino acids with nitriles and α-hydroxynitriles as intermediates. The proposed biosynthetic pathway is shown in Figure 1.3 (Conn, 1979).

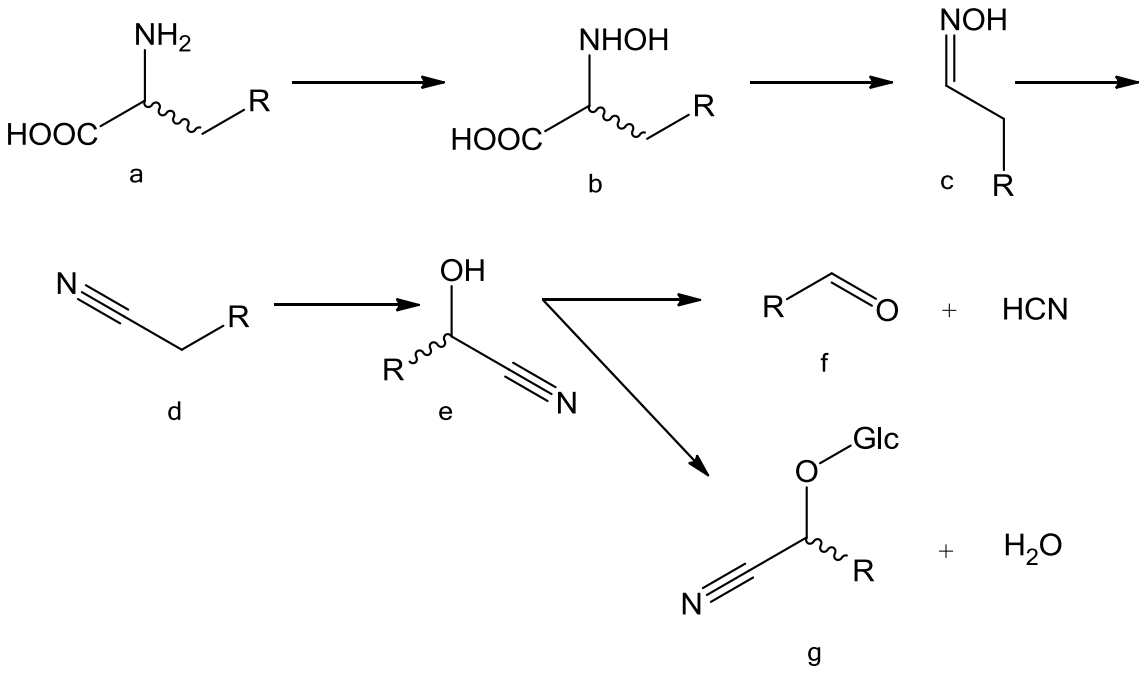


Figure 1.3: Proposed biosynthetic pathway for cyanogenic glycosides: amino acid (a), N-hydroxyamino acid (b), aldoxime (c), nitrile (d), α-hydroxynitrile (e), aldehyde (f), cyanoglycoside (g)

The biosynthesis of glucosinolates which are mostly decomposed into nitriles or isothiocyanates is comprised of three parts, 1) elongation of the amino acid,

2) conversion into a parent glucosinolate, including conjugation with cysteine as the sulfur donor, and 3) secondary modification of the parent glucosinolate (Wittstock and Halkier, 2002).

1.2 Metabolism of nitriles and cyanides

Biodegradation by various microorganisms is an efficient way of removing toxic nitriles and cyanides from the environment. Nitrile-degrading activity has been reported the most in bacteria, the natural role of these enzymes being unclear (Banerjee *et al.*, 2002).

Nitriles and cyanides can be metabolized *via* several pathways (Figure 1.4) (Banerjee *et al.*, 2002).

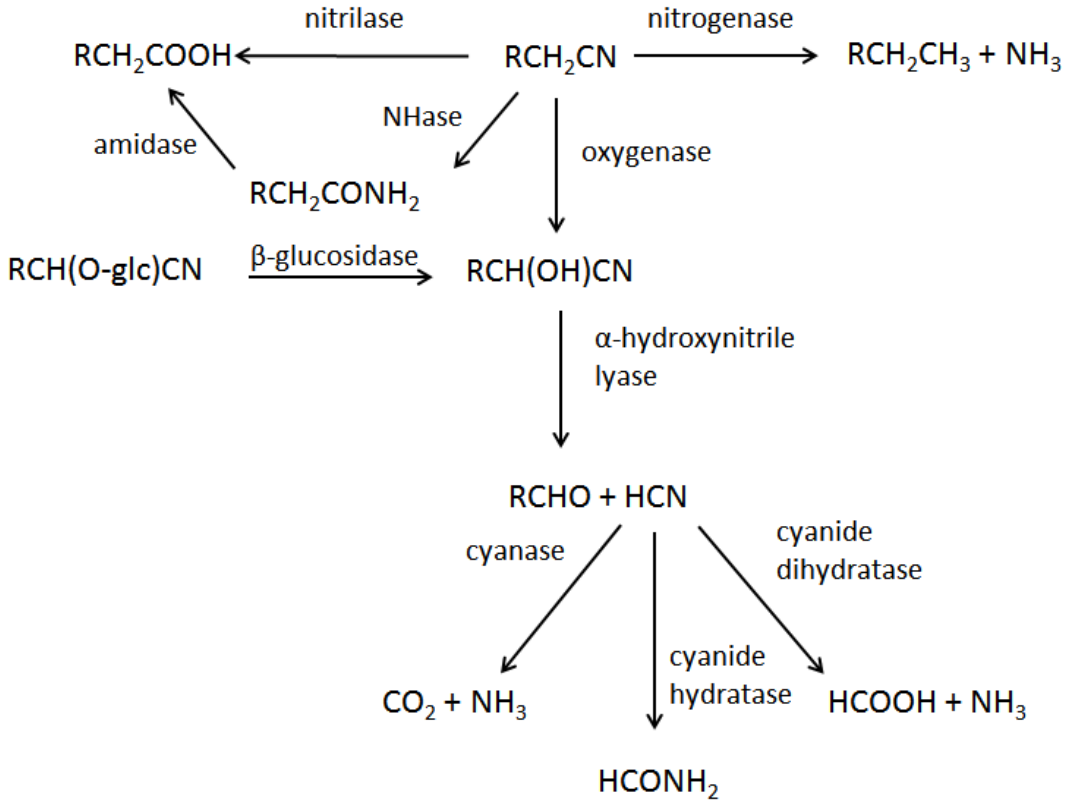


Figure 1.4: Scheme of nitrile and cyanide biodegradation pathways

The reduction of nitriles can be performed by nitrogen fixing organisms, which are capable of reducing molecular nitrogen into ammonia. They can also reduce nitrile by nitrogenase (EC 1.18.6.1; systematic name reduced ferredoxin:dinitrogen

oxidoreductase) under anaerobic conditions. This enzyme is easily inhibited by oxygen (Hardy *et al.*, 1971).

The oxidative conversion of cyanide into carbon dioxide and ammonia is achieved in two different ways, either by cyanide monooxygenase and cyanase (EC 4.2.1.104; systematic name carbamate hydro-lyase) or by cyanide dioxygenase. Both pathways require NADPH (Gupta *et al.*, 2010).

The incorporation of hydrogen cyanide into β-cyano-L-alanine is catalyzed by β-cyano-L-alanine synthase (EC 4.4.1.9; systematic name L-cysteine hydrogen-sulfide-lyase adding hydrogen cyanide, β-cyano-L-alanine-forming). This enzyme replaces the cyanide with the thiol group of a cysteine and produces β-cyano-L-alanine. This cyanide detoxification route is common in plants (Zagrobelny *et al.*, 2004).

The conversion of cyanide into thiocyanate by reaction with sulphur is catalyzed by rhodanese (EC 2.8.1.1; systematic name thiosulfate:cyanide sulfurtransferase). The thiocyanate pathway of cyanide detoxification occurs mainly in vertebrates. The degradation of thiocyanate into sulfate and the corresponding amide is catalyzed by thiocyanate hydrolase (EC 3.5.5.8; systematic name thiocyanate aminohydrolase). Thiocyanate hydrolase has a significant similarity with a bacterial nitrile hydratase (Gupta *et al.*, 2010).

Oxygenases occur in plants and insects and catalyze the conversion of nitriles into cyanohydrins (α-hydroxynitriles), which can be further converted into hydrogen cyanide and aldehyde or keton by hydroxynitrile lyases (Piotrowski, 2008).

The nitrile hydrolysis pathway includes nitrilase or nitrile hydratase and amidase. This is the most common nitrile degradation mechanism. Cyanide dihydratases and cyanide hydratases are the analogues of nitrilases and nitrile hydratases for the conversion of inorganic cyanide.

1.2.1 Nitrile hydratases

Nitrile hydratases (EC 4.2.1.84; systematic name aliphatic amide-hydro lyases) are metalloenzymes requiring iron or cobalt as cofactor and are not members of a nitrilase superfamily (Banerjee *et al.*, 2002). Nitrile hydratases consist of α/β subunits.

The metal cofactor probably enhances the stabilization of the enzyme subunit (Chen *et al.*, 2009).

Nitrile hydratases have only been found in bacteria so far, they are usually produced with amidases in all strains. The final product of this enzymatic pathway is therefore the corresponding carboxylic acid, as with nitrilases. Despite their similar reaction mechanism, nitrilases and nitrile hydratases share low amino acid sequence similarity.

Amidases can be found in prokaryotic and eukaryotic organisms (Banerjee *et al.*, 2002). Although aliphatic amidases form the second branch of the nitrilase superfamily, not all amidases belong to this group. Amidases can also transfer the acyl group in the presence of hydroxylamine and form hydroxamic acids, which can be used in pharmaceuticals (Fournand *et al.*, 1998).

1.2.2 Cyanide hydratases/dihydratases

Cyanide hydratases are closely related to nitrilases; they have not been identified in bacteria or plants, but have been found in many fungal species. In contrast, cyanide dihydratases seem to be purely bacterial in origin (Pace and Brenner, 2001). Cyanide hydratases are closely related to each other, with more than 60 % amino acid identities.

The first cyanide hydratase (EC 4.2.1.66; systematic name formamide-hydro lyase) was discovered in the 1970s, when the tolerance of the fungal phytopathogen *Stemphylium loti* to cyanide and amide formation from hydrogen cyanide were observed (Fry and Millar, 1972). Other cyanide hydratases from the phytopathogenic fungi *Gloeocercospora sorghi* and *Fusarium lateritium* were later purified and characterized (Cluness *et al.*, 1993; Nolan *et al.*, 2003; Basile *et al.*, 2008).

Several enzymes with dual activity have been reported. The cyanide hydratase from *Fusarium oxysporum* N-10 was able to hydrolyze acrylonitrile, crotononitrile and methacrylonitrile (Yanase *et al.*, 2000). Another cyanide hydratase with activity for benzonitrile was found in *Fusarium lateritium* (Nolan *et al.*, 2003). Two recombinant cyanide hydratases from *Aspergillus niger* K10 and *Penicillium chrysogenum*

Wisconsin 54-1255, which were expressed in *E. coli*, exhibited significant activities for 2-cyanopyridine and fumaronitrile, in addition to their high specific activities for hydrogen cyanide (Kaplan *et al.*, 2013).

Cyanide dihydratase (cyanidase; EC 3.5.5.1) forms formate from hydrogen cyanide without formamide as an intermediate in some bacterial strains. Cyanide dihydratase activity has been found for example in *Alcaligenes xylosoxidans* ssp. *denitrificans* (Ingvorsen *et al.*, 1991), in *Bacillus pumilus* C1 (Meyers *et al.*, 1993) or *Pseudomonas stutzeri* AK61 (Watanabe *et al.*, 1998).

Pathogenic fungi produce cyanide hydratases in order to mediate the detoxification of hydrogen cyanide released after injury in their natural hosts, cyanogenic plants (Bork and Koonin, 1994).

1.2.3 Nitrilases

Nitrilases (EC 3.5.5.1; systematic name nitrile aminohydrolases) are enzymes that catalyze the hydrolysis of nitriles (organic cyanides) into the corresponding carboxylic acids and ammonia. They can also form amides as by-products (Fernandes *et al.*, 2006). Nitrilases are able to hydrolyze a broad range of nitrile substrates and have been found in many organisms. Nitrilases can be used for the mild hydrolysis of various nitrile substrates at pH in neutral to slightly alkaline area and ambient temperatures to obtain fine chemicals - valuable carboxylic acids or amides. Other potential uses of nitrilases are the biodegradation of toxic nitriles, detoxification of cyanide waste, or degradation of herbicides such as bromoxynil or chloroxynil (Banerjee *et al.*, 2002).

No posttranslational modifications of nitrilases have been reported to the best of our knowledge, therefore some of the easy and fast prokaryotic expression systems can be used for the production of these enzymes.

1.3 Nitrilase superfamily

Nitrilases belong to the nitrilase family of enzymes acting on non-peptide carbon nitrogen bonds. The nitrilase superfamily consists of 13 branches classified according to sequence similarity but only branch 1 has nitrilase activity; the other

branches have amidase, amide-condensation, acyl transferase or unknown activities (Bork and Koonin, 1994; Pace and Brenner, 2001).

Enzymes in the nitrilase superfamily have various functions such as the synthesis of signaling molecules, vitamin and coenzyme metabolism, detoxification and post-translational modification of proteins. All these enzymes are involved in the reduction of organic nitrogen compounds (Brenner, 2002).

Nitrilases are usually functional as homopolymers, consisting of 4-26 subunits; the reason for forming multimers might be a strong hydrophobicity within the conserved regions (Goldlust and Bohak, 1989; Bork and Koonin, 1994). There are some exceptions to these rules, for example the nitrilase from *Klebsiella ozenae* specific for bromoxynil, along with a nitrilase from *Pyrococcus abyssi*, is active as a dimer (Bhalla *et al.*, 1992; Stalker *et al.*, 1988; Mueller *et al.*, 2006). A nitrilase subunit has a molecular mass of around 40 kDa (32-45) (O'Reilly and Turner, 2003) and a dimer is usually a building block for oligomerization (Thuku *et al.*, 2009).

Nitrilases often form long spiral structures, which can be observed by electron microscopy (Figure 1.5) (Thuku *et al.*, 2009; Vejvoda *et al.*, 2008).

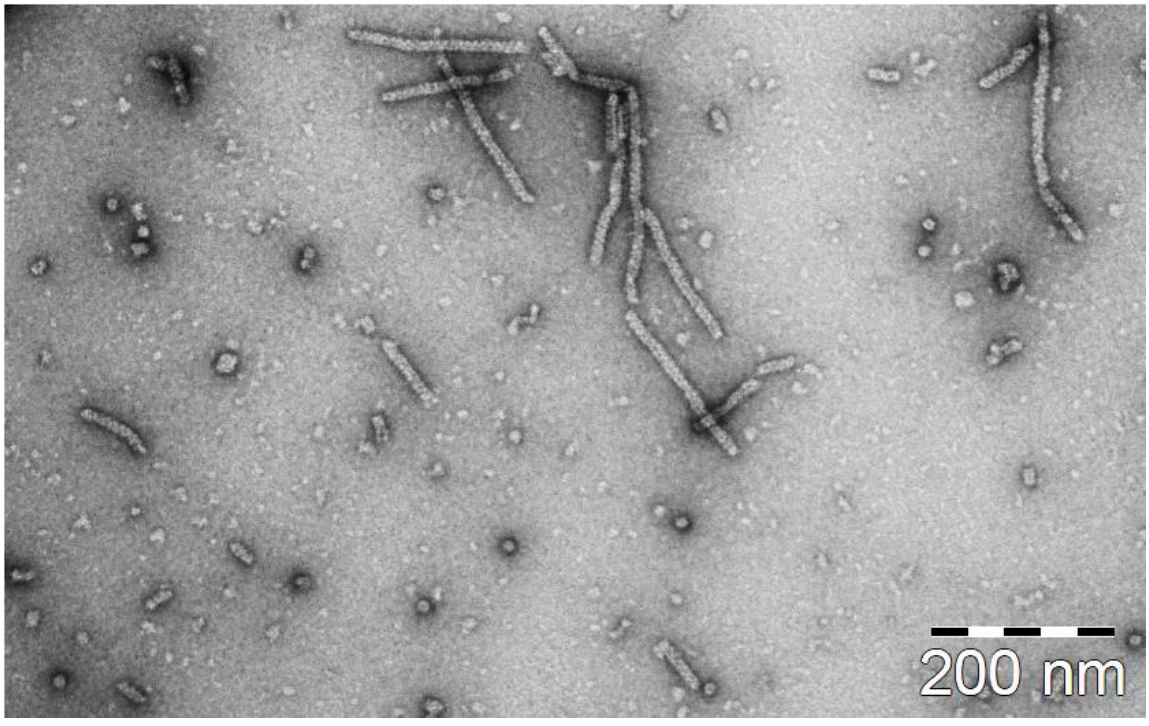


Figure 1.5: Filament-forming purified nitrilases from *Aspergillus niger* K10 observed by electron microscopy (author: O. Benada, Laboratory of Molecular Structure Characterization, Institute of Microbiology, AS CR)

According to the crystal structure of two nitrilase family members – NitFHit from *Caenorhabditis elegans* and *N*-carbamyl-D-aminoacid-amidohydrolase from *Agrobacterium* sp. strain KNK712 – a four-layer sandwich structure αββα and active site consisting of the catalytic triad Glu – Cys – Lys is presumed for all nitrilase family members (Pace *et al.*, 2000; Nakai *et al.*, 2000). In most subfamilies, an additional highly conserved glutamic acid was found, which seems to participate in the reaction mechanism (Thuku *et al.*, 2009; Soriano-Maldonado *et al.*, 2011). The importance of the cysteine residue as part of the catalytic center has been known since 1990s, when inactive mutants were prepared by replacing the cysteine with other amino acids (Kobayashi *et al.*, 1992).

The cysteine residue acts as a catalytic nucleophile, the glutamate residue is a general base (accepting the thiol hydrogen from the Cys residue) and the lysine residue electrostatically stabilizes the tetrahedral intermediate (Nakai *et al.*, 2000). Nitrilases do not require any metal co-factor to function; this fact has been proved by testing with EDTA or potassium cyanide (Layh *et al.*, 1998).

The crystal structure of the thermoactive nitrilase from *Pyrococcus abyssi*, hydrolyzing small aliphatic nitriles such as fumaronitrile and malononitrile, is known, and the structure of its active site has been confirmed (Figure 1.6) (Raczynska *et al.*, 2011).

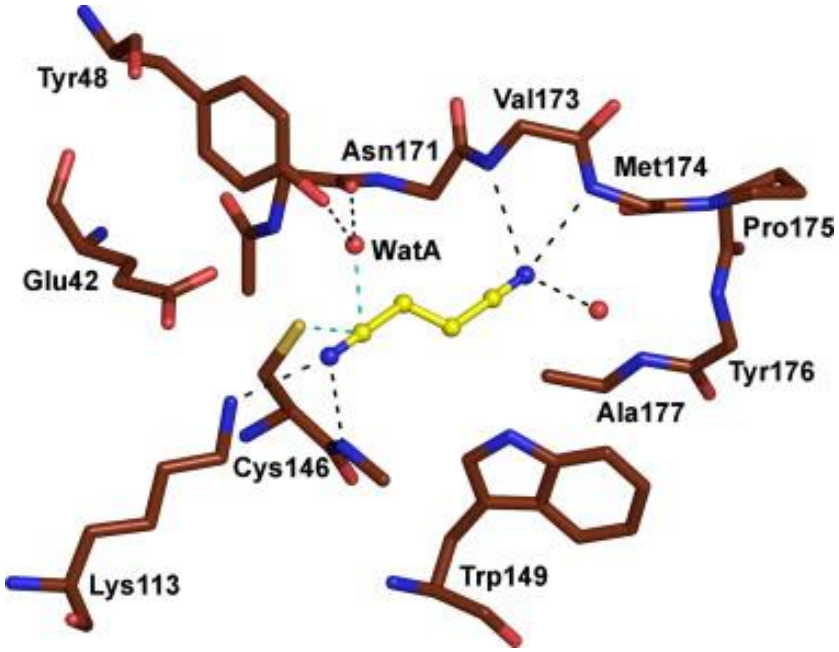


Figure 1.6: *Pyrococcus abyssi* nitrilase active site with docked substrate – fumaronitrile (in yellow) (Raczynska *et al.*, 2011)

1.3.1 Reaction mechanism

The usual product of the nitrilase reaction is the corresponding carboxylic acid and ammonia. Nitrilases can also form an amide as a by-product in some cases. Nitrilases do not require any cofactor or prosthetic group.

Hydrolysis of the substrate (Figure 1.7) begins with nucleophilic attack by a thiol group of the cysteine residue on the carbon atom of a nitrile (**a**); an enzyme-linked tetrahedral thioimidate intermediate is formed (**c**). Attack by a water molecule releases the nitrogen atom as ammonium (**b, d**). The elimination of ammonia requires a positive charge on the nitrogen atom in the reactant, stabilized by the Glu residue. A further molecule of water produces the acid and ensures the regeneration of the enzyme (**e, f**) (Pace and Brenner, 2001; Fernandes *et al.*, 2006).

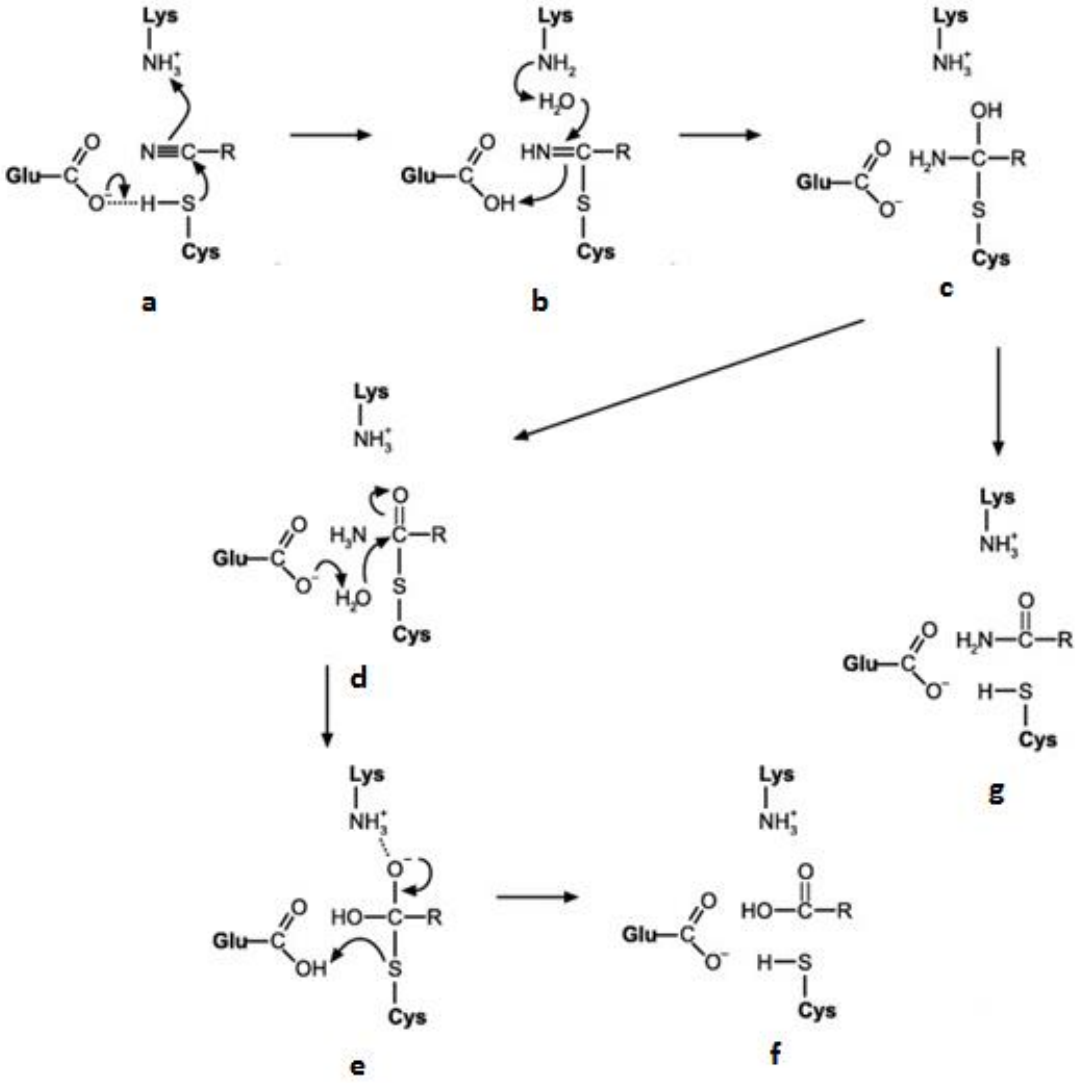


Figure 1.7: Proposed mechanism of nitrile hydrolysis by nitrilase (Piotrowski, 2008)

Thiol binding reagents containing metals such as Ag or Cu seem to be strong inhibitors of nitrilase, confirming the essential role of the cysteine residue for the enzyme activity (Layh *et al.*, 1998; Yamamoto *et al.*, 1991).

Nitrilases can also form the corresponding amide instead of acid (**g**). This effect is expected when the positive charge is not on the reactant, but, for example, on the lysine residue. The amount of amide produced by nitrilases depends on the electron and steric properties of the nitrile substrate and on the structure of the enzyme active site. The amount of amide is higher when the α-substituent is electron-deficient. Also the amount of amide from the (*S*)-enantiomer of (*R,*S)-mandelonitrile is higher than that from the (*R*)-enantiomer (Fernandes *et al.*, 2006).

A high amount of amide is produced by plant nitrilases. A mixture of asparagine and aspartic acid is usually formed from β-cyano-L-alanine (a common substrate of plant nitrilases) (Piotrowski, 2008; Osswald *et al.*, 2002).

1.3.2 Nitrilase occurrence

Nitrilases are widespread in bacterial genera such as *Pseudomonas, Acinetobacter, Alcaligenes, Rhodococcus, Nocardia, Corynebacterium, Comamonas, Pseudomonas, Klebsiella, Bacillus* etc. (Gong *et al.*, 2012; Banerjee *et al.*, 2002).

Nitrile-degrading enzymes were also found in yeasts such as *Pichia, Candida, Saccharomyces, Hanseniaspora, Debaryomyces, Geotrichum, Williopsis, Torulopsis, Exophiala, Kluyveromyces, Aureobasidium, Cryptococcus, Rhodotorula,* but only a few were described as nitrilases (Bhalla *et al.*, 2009; Brewis *et al.*, 1995). These nitrilases could be used in a slightly acidic medium for the hydrolysis of hydroxynitriles and aminonitriles, which are unstable at neutral pH (Gong *et al.*, 2012).

Nitrilases in fungi were described in many strains such as *Fusarium oxysporum* ssp. *melonis*, *Fusarium solani* O1, *Aspergillus niger* K10 or *Penicillium multicolor* CCF 2244 (Goldlust and Bohak, 1989; Vejvoda *et al.*, 2008; Šnajdrová *et al.*, 2004; Kaplan *et al.*, 2006a).

All known fungal nitrilases are inducible like those from bacteria. 2-Cyanopyridine seems to be a good inducer of nitrilases in filamentous fungi; however its expression in suitable hosts is required for biotechnological applications due to the low specific activity of nitrilases from native producers (Martínková *et al.*, 2009).

Generally speaking, the study of fungal nitrilases in their native producers is quite complicated, because of the low production and specific activity of the expressed enzymes.

1.3.3 Substrate specificity of nitrilases

The first nitrile-converting enzyme was found in barley leaves in the 1960s (Thimann and Mahadevan, 1964). This nitrilase catalyzed the conversion of 3-indolylacetonitrile to 3-indolylacetic acid – a plant auxin. It was later found that

3-phenylpropionitrile is a preferred substrate for this enzyme, where the product of this reaction, 3-phenylpropionic acid, also functions as a plant auxin (Vorwerk *et al.*, 2001).

Nitrilases can be divided into several subclasses according to their substrate specificities. Aliphatic nitrilases were found in the genera *Acidovorax, Comamonas, Pseudomonas, Acinetobacter,* etc. (Martínková and Křen, 2010). (Hetero)aromatic nitrilases were found in *Rhodococcus rhodochrous* J1 and in the fungi *Gibberella, Penicillium, Aspergillus,* etc. (Kobayashi *et al.*, 1989; Kaplan *et al.*, 2011). Arylacetonitrilases were described in the strains *Alcaligenes, Pseudomonas, Neurospora, Aspergillus, Nectria, Arthroderma, Halomonas,* etc.; these enzymes usually act enantiospecifically (O'Reilly *et al.*, 2003; Thuku *et al.*, 2009; Chmura *et al.*, 2008; Kaplan *et al.,* 2011; Veselá *et al*., 2013). A nitrilase with specifity for bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) was found in *Klebsiella pneumoniae* ssp. *ozaenae* (Stalker *et al.*, 1988).

In terms of amino acid sequence similarity, nitrilases are closely related to fungal cyanide hydratases and bacterial cyanide dihydratases. However, in contrast to nitrilases which have broad substrate specificities, cyanide hydratases and dihydratases exhibit a strong preference for hydrogen cyanide (O'Reilly and Turner, 2003).

1.3.4 Nitrilase inducers

Nitrilases are in general inducible enzymes, which can be induced by substrates or their analogues (apart from toxic nitriles such as mandelonitrile). For instance, acetonitrile induced nitrilase expression in *Fusarium oxysporum* (Goldlust and Bohak, 1989); isobutyronitrile or isovaleronitrile in *Rhodococcus rhodochrous* J1 (Nagasawa *et al.*, 1988); propionitrile in *Rhodococcus* sp. NDB 1165 (Prasad *et al.*, 2007) or benzonitrile in *Pseudomonas aeruginosa* 10145 (Alonso *et al.*, 2008).

Some constitutive nitrilases have also been found such as those from *Klebsiella ozaenae, Bacillus subtillis* ZJB-063 or *Rhodococcus rhodochrous* J1 (Stalker *et al.*, 1988; Zheng *et al.*, 2008; Kobayashi *et al.*, 1989).

2-Cyanopyridine seems to be a good nitrilase inducer for filamentous fungi, such as those from *Aspergillum, Fusarium* or *Penicillium* (Martínková *et al.*, 2009). In contrast, isovaleronitrile and ε-caprolactam act as powerful nitrilase inducers in rhodococci strains (Nagasawa *et al.*, 1990).

1.4 Industrial use of nitriles and nitrile/cyanide degrading enzymes

Nitriles are finding a broad range of uses in industry as solvents, extractants, pharmaceuticals, drug intermediates (chiral synthons), pesticides and herbicides (bromoxynil, ioxynil, etc.), intermediates in the synthesis of amines, amides, esters, heterocyclic compounds, etc. (Banerjee *et al.*, 2002).

Nitrile pollutants occur in nature as halogenated pesticides (chloroxynil, bromoxynil, ioxynil, etc.) or as organic solvents from industrial processes (acetonitrile, etc.).

Cyanide-containing waste is produced by various industrial processes, such as leach mining, metal finishing and electroplating. A considerable amount of cyanide waste is also produced by processing of cyanogenic crops (cassava or bitter almonds). Cyanide and nitrile detoxification is required to prevent them being deposited into the environment and microbial degradation seems to be a promising method for eliminating these pollutants which proceeds without the further formation of toxic products.

Examples of some chemicals produced by nitrile-hydrolyzing enzymes from nitriles are shown in Figure 1.8.

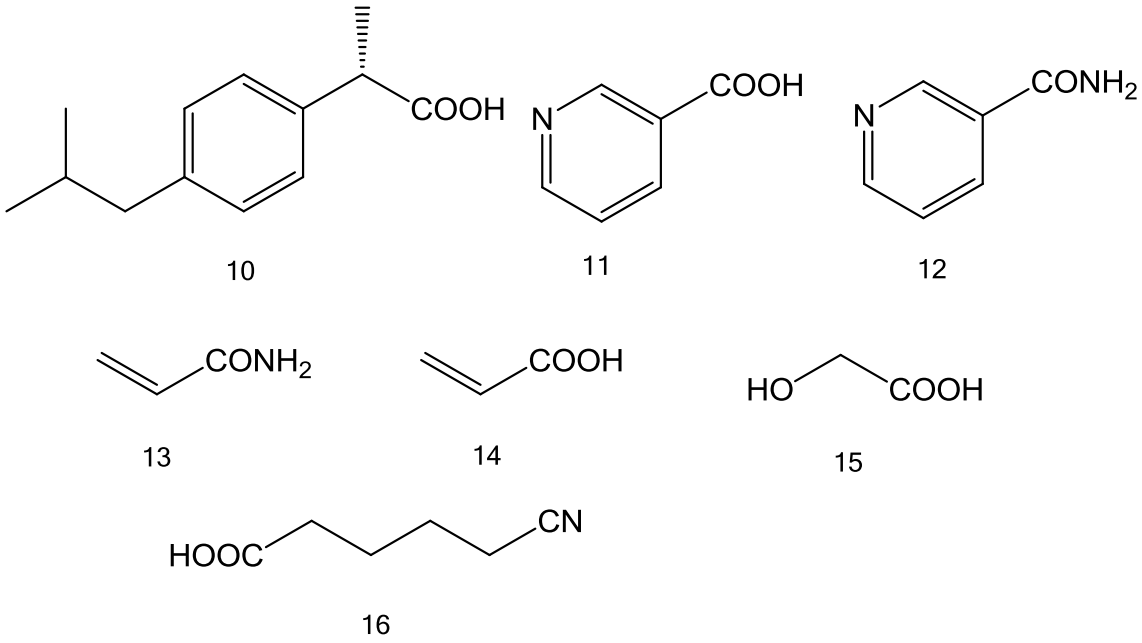


Figure 1.8: Examples of valuable carboxylic acids and amides produced by nitrile-degrading enzymes from nitriles: (*S*)-ibuprofen (10), nicotinic acid (11), nicotinamide (12), acrylamide (13), acrylic acid (14), glycolic acid (15), 5-cyanovaleric acid (16)

An attractive feature of some nitrile-degrading enzymes is their ability to convert chiral nitriles stereoselectively to give optically pure carboxylic acids and amides, such as (*R*)-mandelic acid, (*S*)-ibuprofen (**10**) or optically active amino acids (Yamamoto *et al.*, 1990; Yamamoto *et al.*, 1991; Banerjee *et al.*, 2002).

Compared to nitrile hydratases, nitrilases are usually more stable and enantioselective. Therefore, the ability of some nitrilases to form amide as a by-product can be useful for the production of some industrially important amides.

(*R*)-mandelic acid (**19**), produced from (*R,S*)-mandelonitrile (**17**) by several microorganisms, is a precursor of semi-synthetic cefalosporins and penicillins and a chiral resolving agent (Figure 1.9) (Chen *et al.*, 2009).

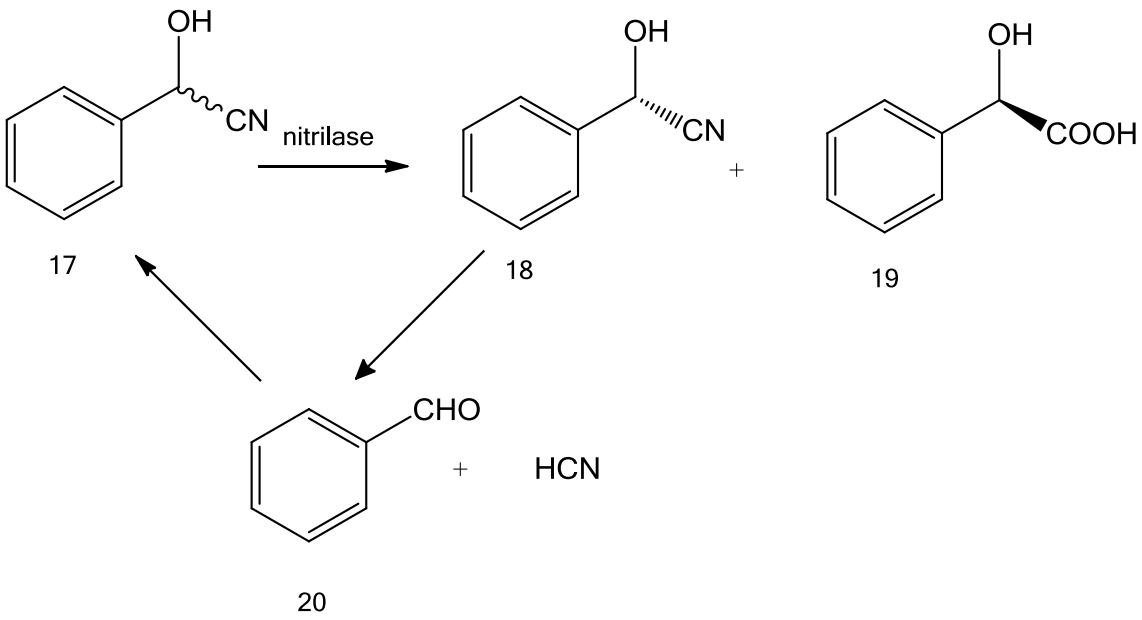


Figure 1.9: Production of (*R*)-mandelic acid by nitrilase from *Alcaligenes faecalis* ATCC8750: (*R,S*)-mandelonitrile (17), (*S*)-mandelic acid (18), (*R*)-mandelic acid (19), benzaldehyde (20) (Yamamoto *et al.*, 1991)

Nicotinamide (**12**) is produced from 3-cyanopyridine using *Rhodococcus rhodochrous* J1 as the catalyst (Lonza AG). Nicotinamide and nicotinic acid (**11**) form part of the water-soluble B-complex vitamins and are used in pharmaceuticals or as additives in food and animal feed (Matthew *et al.*, 1988; Chen *et al.*, 2009).

Nitrile hydratases are used for the production of acrylamide (**13**) from acrylonitrile. Acrylamide is then used for the production of polyacrylamide. Acrylamide is produced on a large scale using *Rhodococcus rhodochrous* J1 as the catalyst (Mitsubishi Rayon Co.) (Yamada and Nagasawa 1990; Gong *et al.*, 2012).

Acrylic acid (**14**) is used in textiles, adhesives and paper treatment and is produced by *Arthrobacter nitroguajacolicus* ZJUTB06-99 from acrylonitrile (Shen *et al.*, 2009).

Glycolic acid (**15**), produced from glycolonitrile, is used in a variety of industrial and medical products and as a monomer in the preparation of polyglycolic acid which is used in the manufacture of dissolvable sutures or drug-delivery and packaging materials (Wu *et al.*, 2008; Panova *et al.*, 2007).

Nitrile-metabolizing enzymes have the ability to selectively hydrolyze one nitrile group in polynitriles, which is rather difficult to achieve using conventional chemical

methods (Bengis-Garber and Gutman, 1989; Kobayashi *et al.*, 1988). *Rhodococcus rhodochrous* K22 is used for the conversion of adiponitrile to 5-cyanovaleric acid (**16**), which is an intermediate in the synthesis of nylon-6 (Godtfredsen *et al.*, 1985).

The majority of industrial applications use nitrilase biocatalysts in the form of immobilized cells or an immobilized enzyme. The advantages of immobilized catalysts are an easier separation of the used enzyme from the reaction mixture and a better stability (Gong *et al.*, 2012).

The usual immobilization methods are entrapment, cross-linking adsorption, covalent bonding or ionic immobilization, the most commonly used immobilization materials being DEAE cellulose, alginate, polyvinyl alcohol, alumina, carrageenan gels or polyacrylamide (Mateo *et al.*, 2004; Gong *et al.*, 2012).

Some of these immobilization methods were used in our laboratoty. CLEAs (cross-linked enzyme aggregates with glutaraldehyde) were used with the nitrile hydratase from *Rhodococcus erythropolis* A4 for the biotransformation of some oxo- and hydroxynitriles (Kubáč *et al.*, 2008). LentiKats® (lense-shaped particles made of polyvinylalcohol-polyethylene glycol copolymer) were used with the lyophilized mycelium of *Fusarium solani* for the mild hydrolysis of 3-cyanopyridine, 4-cyanopyridine and benzonitrile (Vejvoda *et al.*, 2006).

2 Aims of the study

- to find new genes encoding fungal nitrilases in databases
- to express the nitrilases from *Aspergillus niger* CBS 513.88 and *Neurospora crassa* OR74A
    - in native producers
    - in *Escherichia coli*
- to purify and characterize these nitrilases
- to study structural properties of these nitrilases
- to perform site-directed mutagenesis of these nitrilases
- to use these nitrilases in the hydrolysis of a high concentration of (*R,S*)-mandelonitrile

3 Results and discussion

3.1 Genome mining – searching for new genes encoding nitrilases

Most nitrilases have been obtained from cultivable strains isolated by enrichment from soil or by the expression of metagenomic DNA. The former method consists of repeated cultivations of a mixed microbial population with the target compound or its structurally similar analogue (Robertson *et al.*, 2004; Asano, 2002; Martínková *et al.*, 2008).

The screening of metagenomes was performed on the basis of collecting over 600 environmental samples in the work of Robertson *et al.* (2004), 137 new nitrilases were discovered by this approach and characterized (Robertson *et al.*, 2004).

In recent years, genome mining has become very popular. Several nitrilases and cyanide hydratases have been obtained *via* this method, such as the nitrilase from *Synechocystis* sp. (Heinemann *et al.*, 2003), *Pyrococcus abyssi* (Mueller *et al.*, 2006), *Bradyrhizobium japonicum* (Zhu *et al.*, 2007) or *Burkholderia xenovorans* (Seffernick *et al.*, 2009).

We used a genome mining approach to search for new genes encoding putative nitrilases whose catalytic properties were unknown. The gene searches were performed in the GenBank database with the program BLAST (Altschul *et al.*, 1997). Genome mining provided the sequences of putative nitrilase genes in fungi such as *Aspergillus niger* CBS 513.88 and *Neurospora crassa* OR74A (for more details, see Appendix 1). These two enzymes were chosen for further experiments as they are arylacetonitrilases, a group of nitrilases useful for the production of industrially important compounds such as α-hydroxycarboxylic acids.

3.2 Fungal nitrilases in native producers

In the 1960s, several fungal strains (*Aspergillus, Penicillium, Gibberella and Fusarium*) with the ability to convert 3-indolylacetonitrile to 3-indolylacetic acid were reported (Thimann and Mahadevan, 1964).

There have not been many publications about the expression of fungal nitrilases compared to those concerned with bacterial nitrilases, and only a few fungal nitrilases have been purified and characterized. The purified nitrilase from *Fusarium solani* IMI196840 was able to hydrolyze benzonitrile, benzo-1,4-dinitrile, 4-cyanopyridine and some other (hetero)aromatic nitriles (Harper, 1977); and the nitrilase from *Fusarium oxysporum* ssp. *melonis* was able to hydrolyze nitriles including benzonitrile, acrylonitrile and crotononitrile (Goldlust and Bohak, 1989).

The strains *Aspergillus niger* K10, *Fusarium solani* O1, *Fusarium oxysporum* CCF 1414, *Fusarium oxysporum* CCF 483 and *Penicillium multicolor* CCF 2244 were able to hydrolyze benzonitrile or cyanopyridines after cultivation in Czapek-Dox medium with addition of 2-cyanopyridine (Kaplan *et al.*, 2006a). The nitrilases from *Aspergillus niger* K10 and *Fusarium solani* O1 were then purified and characterized (Kaplan *et al.*, 2006b; Vejvoda *et al.*, 2008).

The purified nitrilase from *Fusarium solani* IMI196840, induced by 2-cyanopyridine, had similar substrate specificity to the nitrilase isolated from the same strain previously by Harper in 1977, but with an approximately 380-fold higher activity for benzonitrile (Vejvoda *et al.*, 2010).

In our experiments, the fungal strains *Aspergillus niger* CBS 513.88 (#FGSC A1513) and *Neurospora crassa* OR74A (#FGSC 9013) (McCluskey, 2003) were tested for the expression of nitrilases in the wild-type producing strains.

The strains were cultivated in Czapek-Dox medium for 48 - 168 hours. Various compounds were used as potential inducers – potassium cyanide, acetonitrile or 2-cyanopyridine, which was determined as the best inducer from previous experiments (Kaplan *et al.*, 2006a).

A. niger and *N. crassa* strains grew well on 2-cyanopyridine and acetonitrile and poorly on potassium cyanide.

The nitrilase activity in the fungal mycelium was examined with phenylacetonitrile as substrate. The highest amount of product (phenylacetic acid) was observed for the *N. crassa* strain induced by 2-cyanopyridine, when cultivated for 3 days (0.66 mM of phenylacetic acid per gram of wet mycelium per hour). The *A. niger* strain produced a similar amount of phenylacetic acid after induction by 2-cyanopyridine or acetonitrile, when cultivated for 7 days (an approximately 1.2-fold lower amount of product than *N. crassa*). No nitrilase or cyanide hydratase activity was observed after potassium cyanide induction with any of the fungal strains.

In order to enable a defined amount of fungal material to be used, the mycelium was lyophilized or disintegrated in a mortar. Unfortunately, both methods led to a complete loss of activity. As a result, isolating the protein from the mycelium was not possible either.

The cultivation conditions were optimized towards a higher biomass yield in *Neurospora crassa*. Rich medium (sucrose 30 g/l, malt extract 5 g/l, yeast extract 5 g/l, $NaNO_3$ 2 g/l) was used in the first cultivation step (30 h). In the second step (18 h), Czapek-Dox medium without sucrose, containing an inducer (2-cyanopyridine or acetonitrile, 2 g/l each) was used according to a method which previously proved useful in *Fusarium* strains (Vejvoda *et al.*, 2006). Some other inducers were also tested – 4-hydroxyphenylacetonitrile, 3-indolylacetonitrile, and 2-cyanopyridine at higher concentration (3 g/l).

This cultivation method led to a significantly higher production of the mycelium, but the nitrilase activity was still very low.

A higher concentration of the inducer (3 g/l compared to 2 g/l) led to a 20-% increase in the relative activity of the mycelium.

In recent years, nitrilases from various organisms, both prokaryotic and eukaryotic, were overexpressed in suitable hosts such as *Escherichia coli* in order to obtain sufficient amounts of active enzyme.

3.3 Expression of fungal nitrilases in *Escherichia coli*

A huge number of bacterial nitrilases have been successfully cloned and overexpressed in *E. coli* so far (e.g., Lévy-Schil *et al.*, 1995; Kiziak *et al.*, 2005; Banerjee *et al.*, 2009; Zhang *et al.*, 2011); the first was the bromoxynil-degrading nitrilase from *Klebsiella pneumoniae* ssp. *ozaenae* (Stalker *et al.*, 1987).

Several fungal nitrilases were expressed in *E. coli* in recent years by our group, such as the nitrilases from *Aspergillus niger*, *Neurospora crassa, Gibberella moniliformis* or *Penicillium marneffei* (Kaplan *et al.*, 2011; Petříčková *et al.*, 2012); nitrilases from *Arthroderma benhamiae* and *Nectria haematococca* (Veselá *et al.*, 2013) or nitrilases from *Aspergillus oryzae* or *Meyerozyma guilliermondi* (Kaplan *et al.*, 2013). Several cyanide hydratases, from *Aspergillus nidulans, Neurospora crassa, Gibberella zeae* and *Gloeocercospora sorghi*, were also expressed in *E. coli* (Basile *et al.*, 2008).

Heterologous expression in *Escherichia coli* seems to be an easy and fast way to produce nitrilases in large quantities. The recombinant nitrilases were confirmed to be also suitable for our work and were used in further experiments.

The nitrilases from *Aspergillus niger* CBS 513.88 (GenBank: CAK46742) and *Neurospora crassa* OR74A (GenBank: CAD70472) were expressed in *E. coli* at high levels (23 000 and 69 000 units per liter, respectively).

These two nitrilases exhibited less than 50 % amino acid identity to each other (for more details, see Appendix 2). Two other nitrilases from *Aspergillus* (*Aspergillus niger* CBS 513.88 = *A. niger 2*, GenBank: CAK47246; *Aspergillus oryzae* RIB40, GenBank: BAE63579) were later also expressed, which shared 50 – 60 % amino acid identities to the nitrilases from *N. crassa* and *A. niger* (for the amino acid sequences of the expressed nitrilases, see Supplementary figure S2).

All of the recombinant fungal nitrilases produced were only distantly related to well-known bacterial nitrilases, with approximately 40 % identity.

All *E. coli* strains expressing the fungal nitrilases were tested with various (hetero)aromatic, aliphatic and arylaliphatic substrates – phenylacetonitrile (**21**), (*R,*S)-mandelonitrile (**22**), (*R,S*)-2-phenylpropionitrile (**23**), 3-phenylpropionitrile (**24**), benzonitrile (**25**), 2-cyanopyridine (**26**), 3-cyanopyridine (**27**), 4-cyanopyridine (**28**),

3-indolylacetonitrile (**29**), fumaronitrile (**30**), propionitrile (**31**), valeronitrile (**32**), (4*S*,5*R*)-2,4-diphenyl-5,6-dihydro-4H-1,3-oxazine-5-carbonitrile (**33**) (taxol precursor) (Figure 3.1).

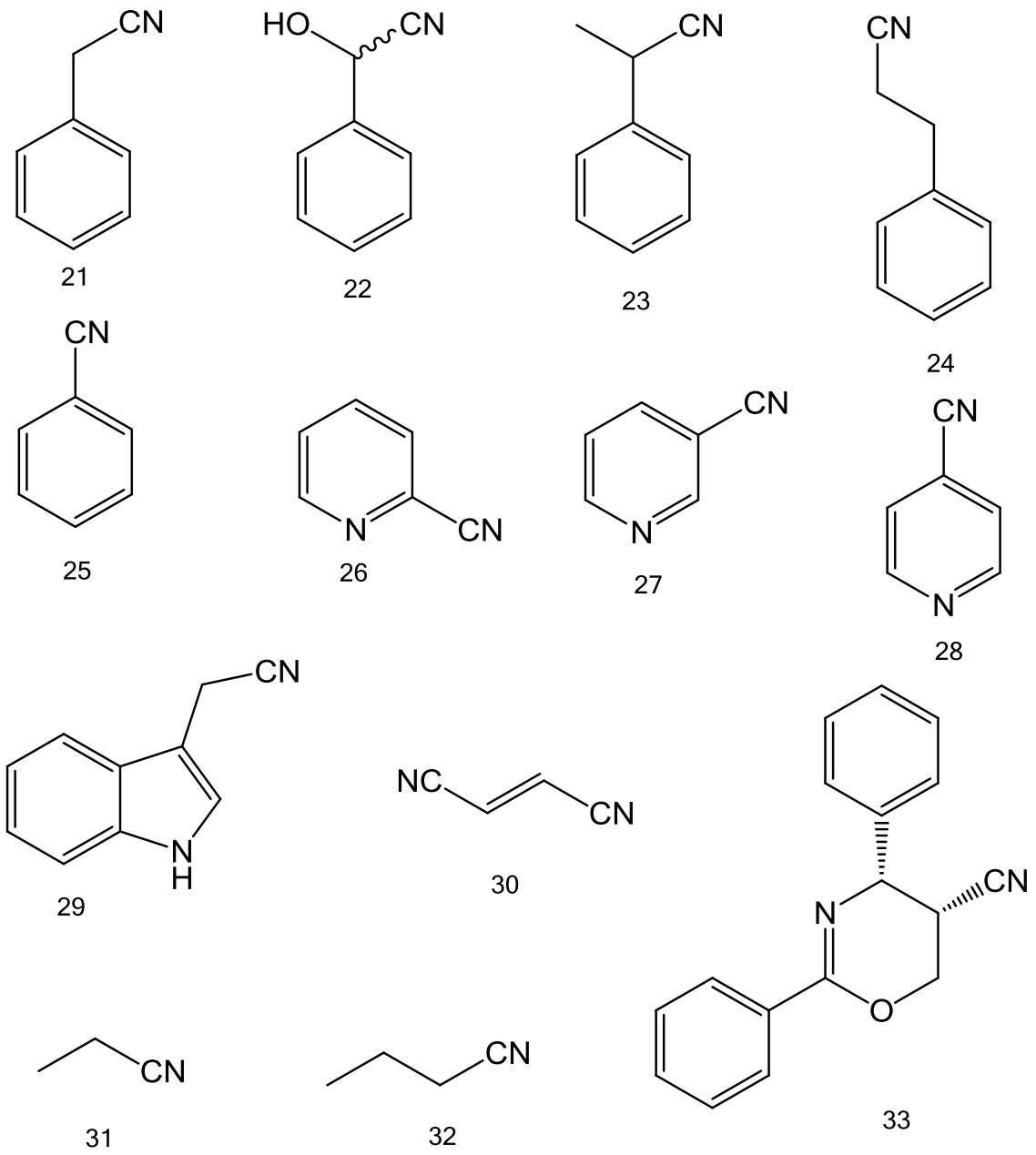


Figure 3.1: (Hetero)aromatic, aliphatic and arylaliphatic nitriles examined as nitrilase substrates: phenylacetonitrile (21), (*R,*S)-mandelonitrile (22), (*R,*S)-2-phenylpropionitrile (23), 3-phenylpropionitrile (24), benzonitrile (25), 2-cyanopyridine (26), 3-cyanopyridine (27), 4-cyanopyridine (28), 3-indolylacetonitrile (29), fumaronitrile (30), propionitrile (31), valeronitrile (32), and (4*S*,5*R*)-2,4-diphenyl-5,6-dihydro-4*H*-1,3-oxazine-5-carbonitrile (33) (a taxol precursor; kindly provided by Dr. Norbert Klempier and Ing. Birgit Wilding (TU Graz)).

E. coli strains producing the nitrilases from *N. crassa*, *A. niger* and *A. oryzae* were compared in terms of their substrates preferences. All of them exhibited the highest activity for phenylacetonitrile and (*R,S*)-mandelonitrile. Significant activity for

3-indolylacetonitrile was also observed with all strains (Figure 3.2). All enzymes exhibited low activities for aliphatic and aromatic substrates (Figure 3.3). The only nitrilase with a slight activity for potassium cyanide was the nitrilase from *N. crassa* (for more details, see Appendix 4).

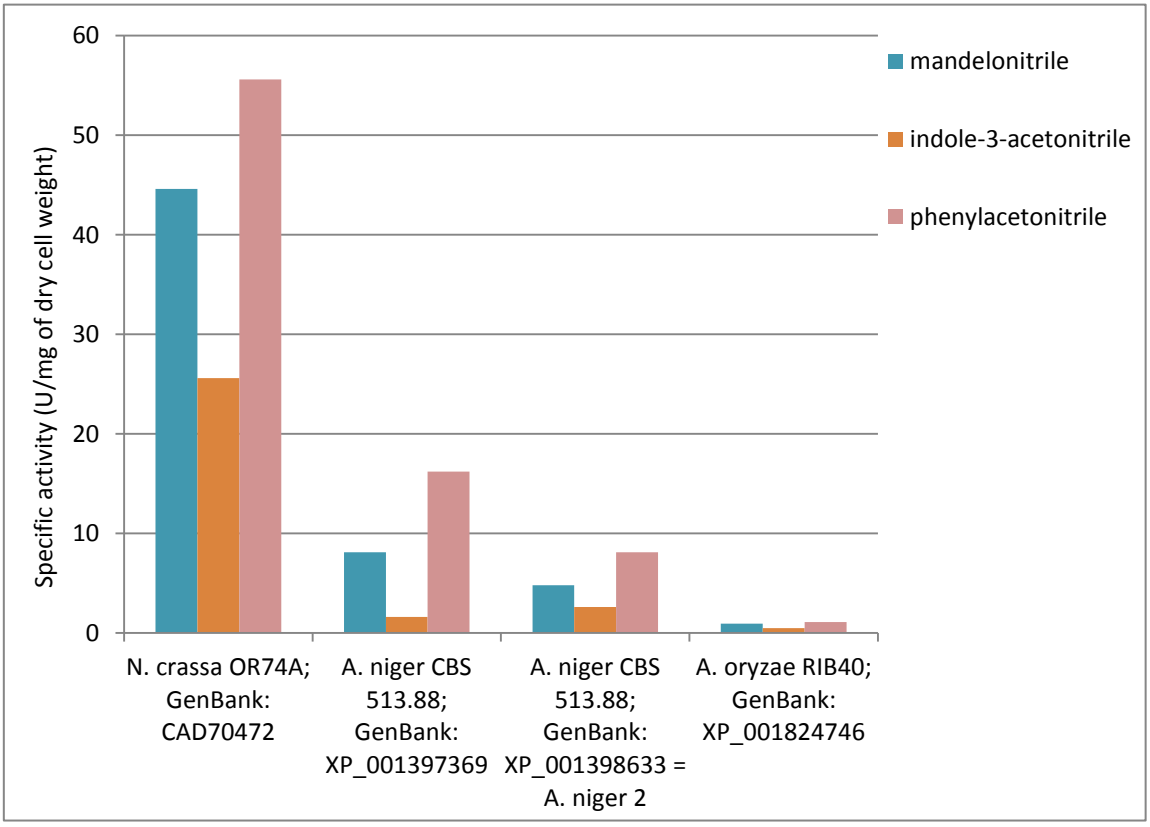


Figure 3.2: Comparison of specific activities of arylacetonitrilases for preferred substrates.

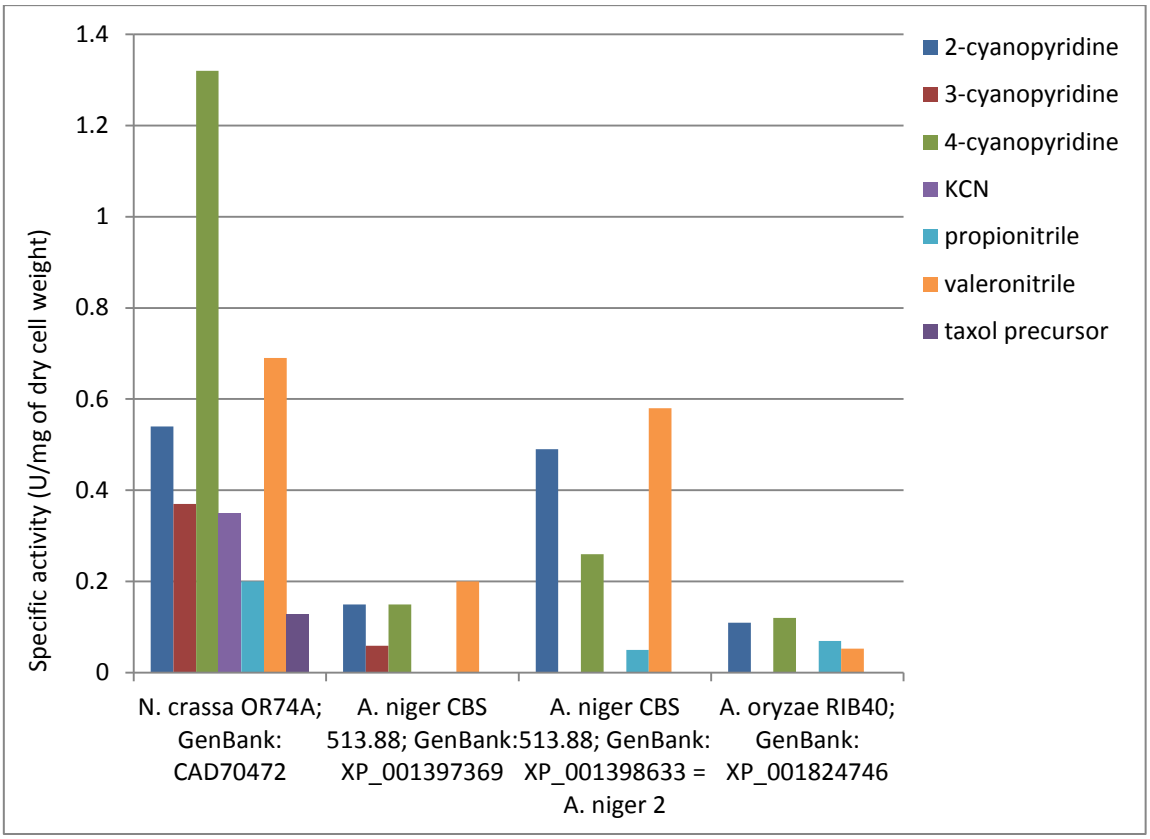


Figure 3.3: Comparison of specific activities of arylacetonitrilases for poor substrates. Activities of less than 0.05 U/mg of dry cell weight are not displayed.

The nitrilases from *Aspergillus niger* CBS 513.88 and *Neurospora crassa* OR74A were the first reported recombinant fungal arylacetonitrilases with activity for phenylacetonitrile and other arylaliphatic substrates.

Arylacetonitrilases can be generally used for the production of some optically active compounds such as (*S*)-(+)-ibuprofen from (*R,S*)-(±)-ibuprofen by *Acinetobacter* sp. strain AK226 – in approximately 95% e.e. (Yamamoto *et al.*, 1990). (*R*)-mandelic acid was formed with high enantioselectivity from (*R,*S)-mandelonitrile, for example by the nitrilases from *Alcaligenes faecalis* ATCC 8750, *Alcaligenes faecalis* JM3, *Pseudomonas putida* or *Microbacterium paraoxydans* (Yamamoto *et al.*, 1991; Nagasawa *et al.*, 1990, Banerjee *et al.*, 2009; Kaul *et al.*, 2004). (*R*)-Mandelic acid is also produced commercially by Mitsubishi Rayon Co. along with (*R*)-chloromandelic acid (Brady *et al.*, 2004).

A recombinant whole-cell catalyst producing the arylacetonitrilase from *Pseudomonas fluorescens* EBC191 together with the oxynitrilase from cassava (*Manihot*

esculenta) formed (*S*)-enantiomers of mandelic acid and mandelamide from (*R,S*)-mandelonitrile (Sosedov *et al.*, 2009).

3.4 Purification and characterization of recombinant arylacetonitrilases

The codon frequency was optimized for expression in *E. coli*. The nitrilase from *N. crassa* was used for a comparison of two different optimization methods (from GeneArt, Germany; and Generay, China) and expression of a non-optimized gene (for the nucleotide sequences of the *N. crassa* genes, see Supplementary figure S1). The enzymes were expressed from all genes and the specific activities decreased in the order: gene optimized by GeneArt > gene optimized by Generay > non-optimized enzyme. The level of expression was similar in all cases.

All nitrilase genes were cloned in the *Nde*I and *Hind*III sites of the pET 30a(+) vector and expressed in *E. coli* BL21 gold (DE3). Strains were cultivated with kanamycin (100 μg/ml) at 30 °C for 3 h, nitrilase expression was induced with IPTG (0.5 mM) and the cultivation continued for 18 h.

The effect of the N-terminal and C-terminal His_6-tag was examined with the same nitrilase from *N. crassa*. The highest specific activity was obtained with a variant without the His_6-tag. Moreover, both His_6-tagged variants were unstable, according to the low yields of purification of these fused proteins (1 and 1.6 % yield for the N-terminal and C-terminal His_6-tagged variants, respectively), which could be caused by precipitation of the protein during purification. Therefore, the purification method needs to be optimized to enable further use of these nitrilase variants.

The level of nitrilase expression was similar in all cases (Figure 3.4).

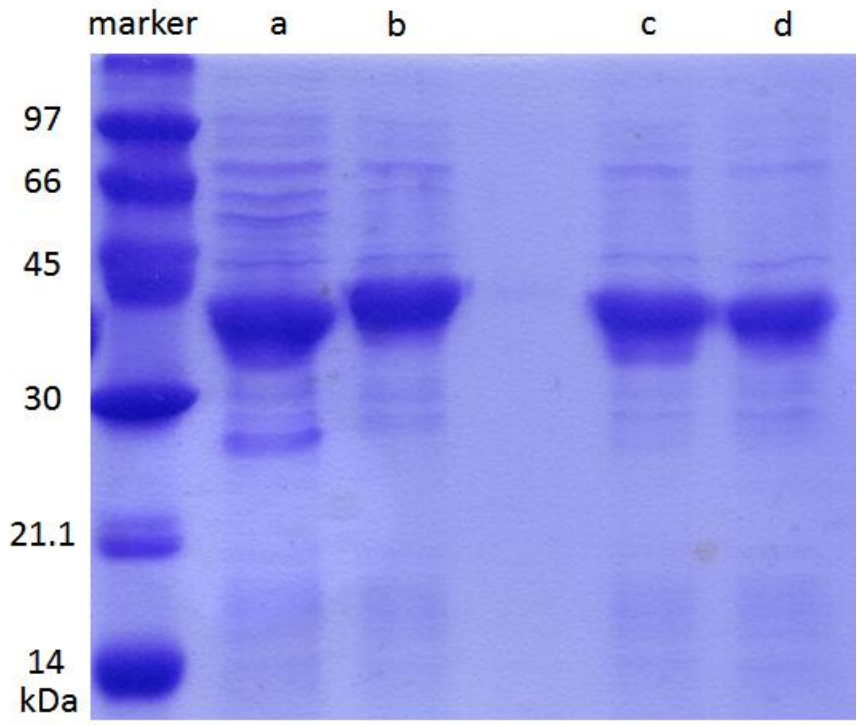


Figure 3.4: Comparison of protein expression level of various nitrilase variants from *N. crassa:* sequence optimization by GeneArt (a), sequence optimization by Generay + N-terminal His_6-tag (b), sequence optimization by Generay (c), no sequence optimization (d)

The nitrilase variants with a sequence optimization were subsequently produced on a larger scale and purified (for more details, see Appendix 2). The enzymes from *N. crassa* and *A. niger* formed 42 % and 33 % of the total soluble protein, respectively.

The resting cells of *E. coli* were disrupted by sonication. The nitrilase was purified from the cell-free extract by ion exchange chromatography in a Hi-Prep 16/10 Q FF column in a linear gradient of NaCl (0.15 – 1 M). Active fractions were pooled, concentrated and applied to a Superdex 200 column. Nitrilases from both strains were purified with approximately 50 % yields (Table 3.1; Table 3.2; Figure 3.5).

Table 3.1: Purification of nitrilase from *Aspergillus niger* CBS 513.88

	$A_{spec.}$ (U/mg of protein)	A_{total} (U)	Yield (%)
Cell free extract	10.6	822.9	100.0
After ion exchange chromatography	15.6	589.4	71.6
After gel filtration	31.5	412.8	50.2

Table 3.2: Purification of nitrilase from *Neurospora crassa* OR74A

	$A_{spec.}$ (U/mg of protein)	A_{total} (U)	Yield (%)
Cell free extract	25.2	602.3	100.0
After ion exchange chromatography	32.6	392.5	65.2
After gel filtration	59.9	295.3	49.0

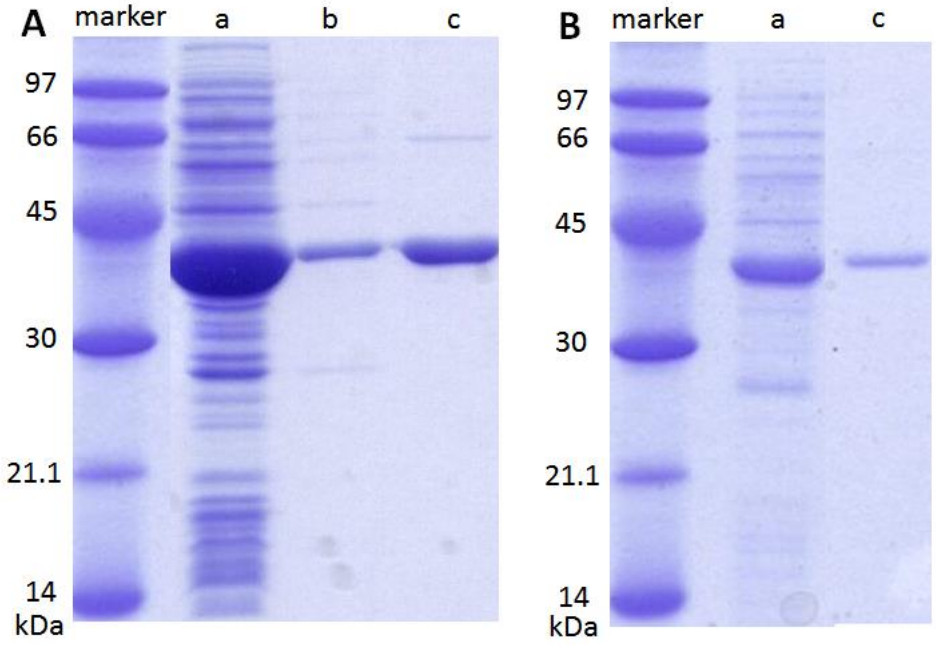


Figure 3.5: SDS PAGE *Aspergillus niger* (A), *Neurospora crassa* (B); cell free extract (a), after ion exchange chromatography (b), after gel filtration (c)

His_6-tagged nitrilase variants were purified by affinity chromatography in a HisPrep FF 16/10 column with a linear gradient of imidazole (0.03 – 0.5 M).

Nitrilase activity was assayed with 25 mM phenylacetonitrile. The nitrilase activity was calculated in the linear range of the reaction, from the concentration of reaction products detected by HPLC using a PDA detector at 210 nm.

The role of molecular chaperones was examined in the aforementioned nitrilases. Only in strains where GroEL/ES and trigger factor were expressed was a slight increase in enzyme solubility and activity observed (1.25-fold) (for more details, see Appendix 2).

The activities of the nitrilases were tested with the same set of substrates as whole cells of *E. coli* producing the respective enzymes. The experiments with purified proteins confirmed the results obtained by experiments with whole cells. The biocatalytic properties, such as substrate specificity or amide formation, of both biocatalyst systems (whole cells of *E. coli* producing nitrilase vs. purified enzyme) were similar.

The purified nitrilases had a similar V_{max} value with (*R,S*)-mandelonitrile and phenylacetonitrile, but their K_m values for phenylacetonitrile were significantly lower than for mandelonitrile (Table 3.3). The K_m value for 2-phenylpropionitrile was the lowest for both enzymes, but both nitrilases also had the lowest V_{max} for this substrate (30-175 times lower than with phenylacetonitrile).

Table 3.3: Kinetic parameters of nitrilases from *Aspergillus niger* and *Neurospora crassa.* PAN = phenylacetonitrile, (*R,*S)-MN = (*R,*S)-mandelonitrile, (*R,S*)-2-PPN = (*R,*S)-2-phenylpropionitrile

	Aspergilllus niger		***Neurospora crassa***	
	K_m (mM)	**V_{max} (U/mg of protein)**	**K_m (mM)**	**V_{max} (U/mg of protein)**
PAN	3.4	10.6	2.0	17.5
(*R,S*)-MN	11.4	12.4	3.4	9.9
(*R,S*)-2-PPN	0.8	0.4	1.3	0.1

The pH optima of previously reported nitrilases are mostly in the slightly alkaline range 7 to 9.5 (Gradley *et al.*, 1994; Stalker *et al.*, 1988; Bhalla *et al.*, 1992). In contrast, the nitrilase from *Rhodococcus rhodochrous* K22 exhibited maximum activity at pH 5.5

(Kobayashi *et al.*, 1990) and the nitrilase from *Arthrobacter nitroguajacolicus* ZJUTB06-99 had a pH optimum 6.5 (Shen *et al.*, 2009). The nitrilases from *A. niger* and *N. crassa* exhibited their highest activities at pH 9 (Figure 3.6).

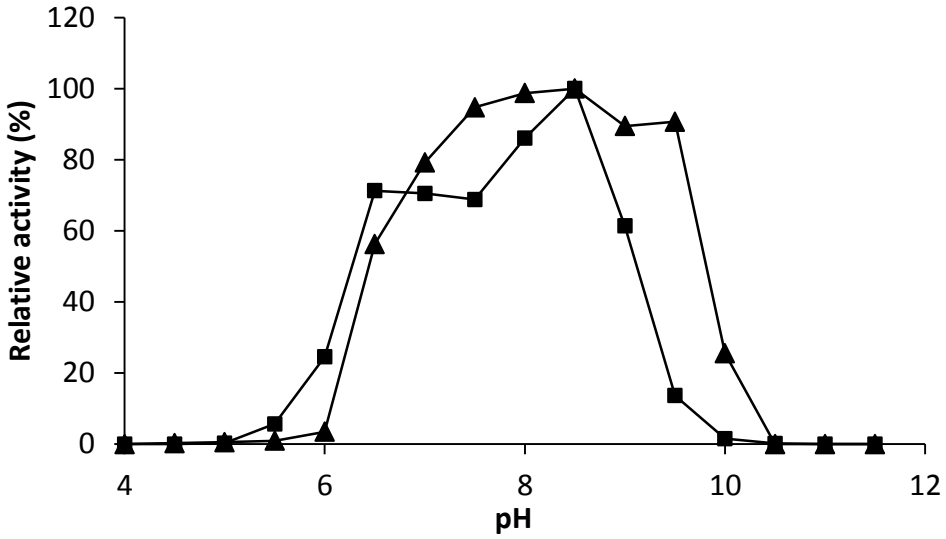


Figure 3.6: Optimal pH values for nitrilase activity: *A. niger* nitrilase (▲), *N. crassa* nitrilase (■)

The temperature optima in the previously studied nitrilases ranged between 30 and 55 °C (Zhang *et al.*, 2001; Layh *et al.*, 1998; Nagasawa *et al.*, 1990). An exception is the highly thermostable nitrilase from *Pyrococcus abyssi* GE5, which exhibited the highest activity at 80 °C (Mueller *et al.*, 2006). Both nitrilases examined by ourselves had temperature optima of 38 °C (Figure 3.7).

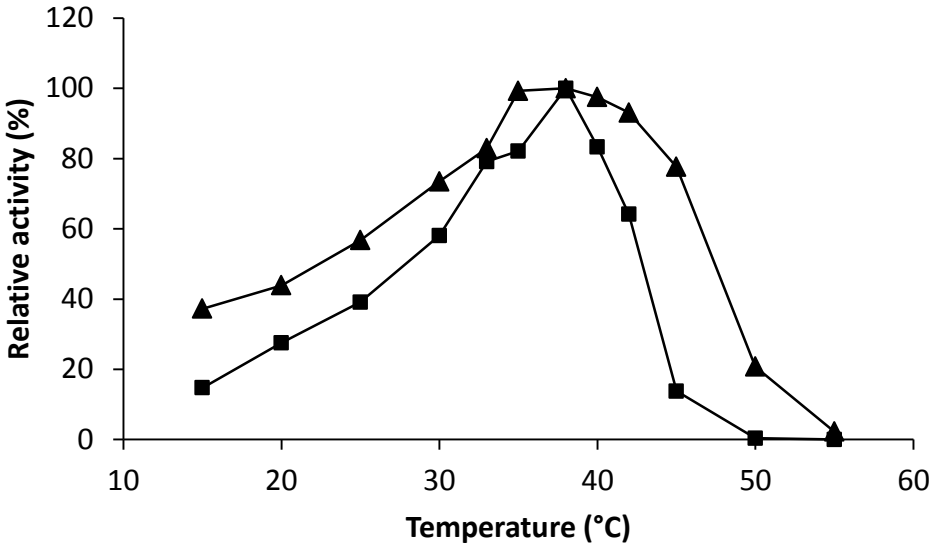


Figure 3.7: Optimal temperatures for nitrilase activity: *A. niger* nitrilase (▲), *N. crassa* nitrilase (■)

Enzyme stability is a key issue for industrial-scale biocatalysis.

Both the nitrilase from *A. niger* and *N. crassa* were stable at temperatures below 35 °C, similar to the nitrilase from *Rhodococcus rhodochrous* PA-34 or from *Alcaligenes faecalis* ZJUTB10 (Bhalla *et al.*, 1992; Xue *et al.*, 2011). In contrast, the nitrilase from the extremophile *Pyrococcus abyssi* was stable in the temperature range 60 to 90 °C, with a half-life of 9 h at 80 °C (Mueller *et al.*, 2006).

The nitrilase from *A. niger* and *N. crassa* were stable in the pH range 7.0 – 10.0 and 6.5 – 9.0, respectively (after 2-h preincubation at room temperature at respective pH), which is similar to previously examined nitrilases (for more details, see Appendix 2).

3.5 Structural properties of nitrilases

Electron microscopy is an important method in nitrilase studies due to the lack of available crystal structures of these enzymes. In recent years, several nitrilases have been studied in this way. The fungal nitrilase from *Fusarium solani* formed extended complex rods up to 500 nm long, or aggregates. The nitrilase from *Aspergillus niger* K10 also formed rods up to 250 nm long, but no aggregates were observed (see Figure 1.5 in section 1.3) (Vejvoda *et al.*, 2008). Similar structures were also observed for the

cyanide dihydratase from *Bacillus pumilus* (Jandhyala *et al.*, 2003). In contrast, the purified nitrilases from *A. niger* and *N. crassa* formed typical high molecular weight structures, but no filamentous forms (Figure 3.8, Figure 3.9; the electron micrographs were provided by Dr. O. Benada, Institute of Microbiology, AS CR).

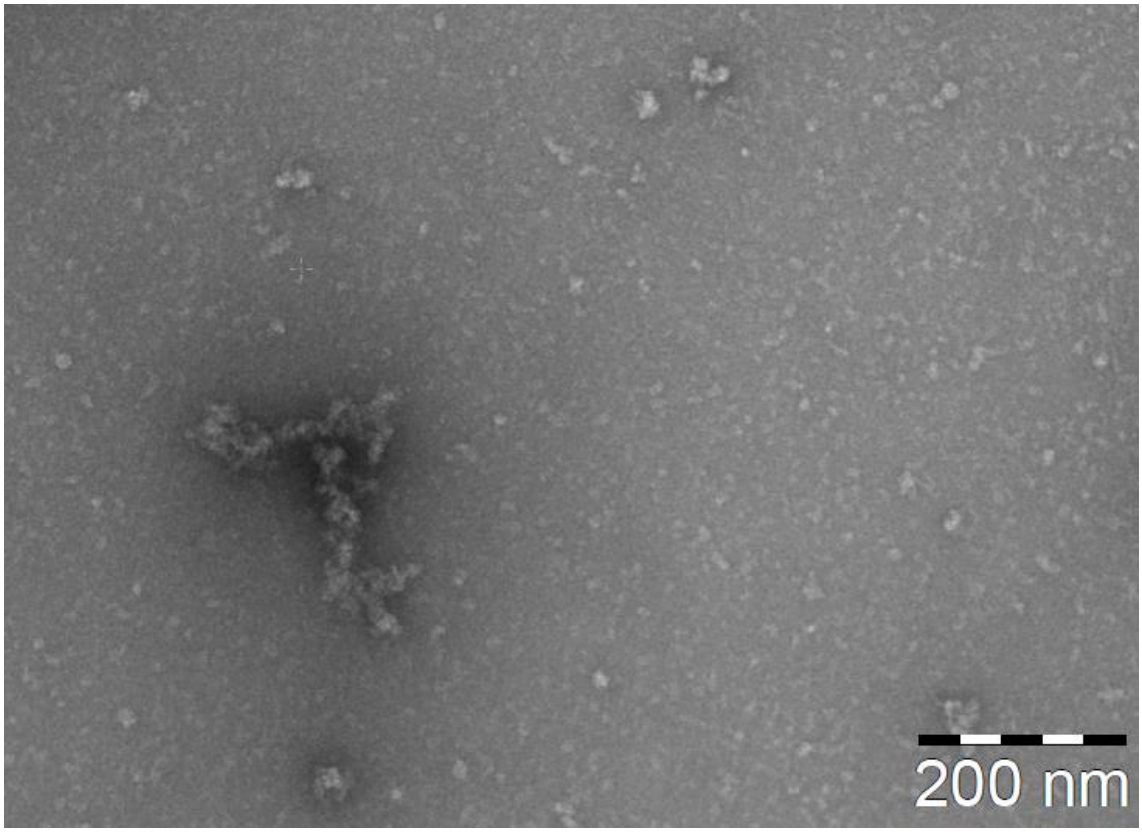


Figure 3.8: Purified nitrilase from *Aspergillus niger* CBS 513.88 observed by electron microscopy (author: Dr. O. Benada, Laboratory of Molecular Structure Characterization, Institute of Microbiology, AS CR)

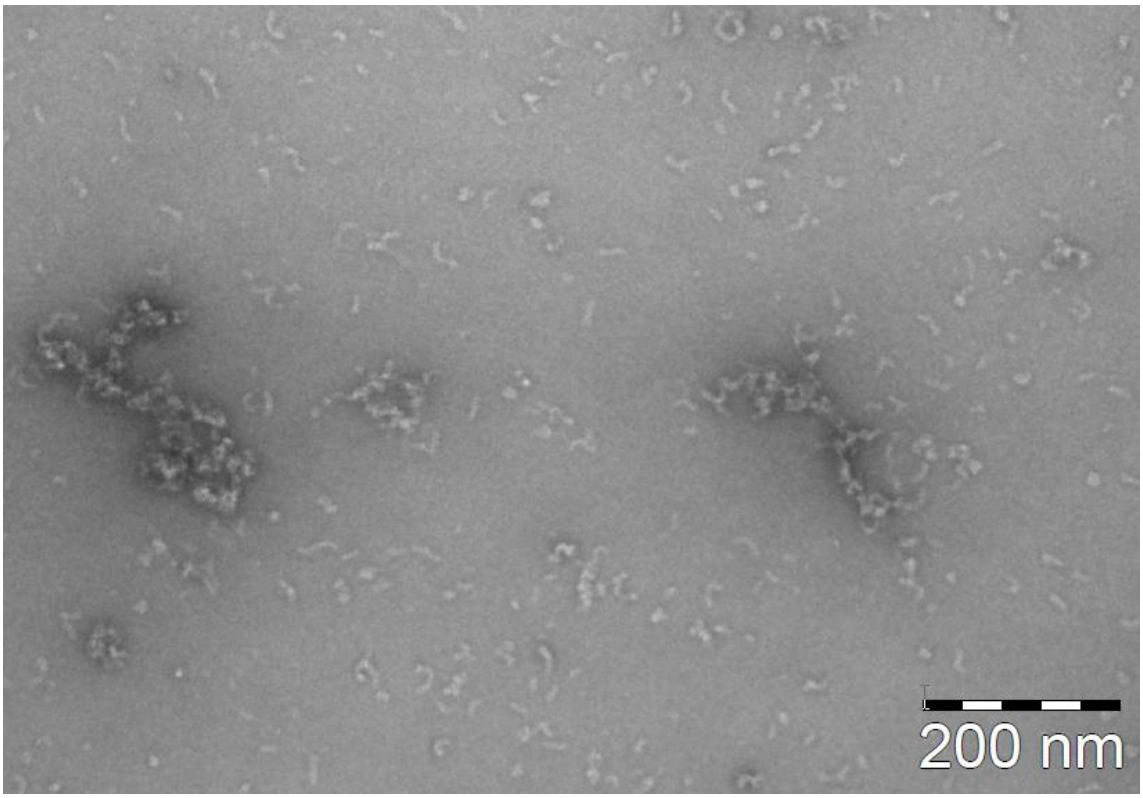


Figure 3.9: Purified nitrilase from *Neurospora crassa* OR74A observed by electron microscopy (author: Dr. O. Benada, Laboratory of Molecular Structure Characterization, Institute of Microbiology, AS CR)

Another important method – homology modelling – was used for protein modelling based on their homology to evolutionarily related proteins, where the protein with known amino acid sequence is compared to a homologous protein with a known three-dimensional structure (Kaczanowski and Zielenkiewicz, 2010).

Three different templates were used to obtain a homology model of *N. crassa* nitrilase – 2w1v (mouse nitrilase), 1j31 (*Pyrococcus horikoshi* nitrilase) and 1fo6 (amidohydrolase from *Agrobacterium radiobacter*), with less than 30 % amino acid sequence similarity to the examined nitrilase (for the multiple sequence alignment, see Supplementary figure S3) (the homology modelling was made by Dr. N. Kulik, Institute of Nanobiology and Structural Biology, AS CR).

A typical nitrilase four-layered αββα structure was observed with long loops close to the active site (Figure 3.10).

The hexapeptide, which only occurs in fungal nitrilases, is positioned far from the entrance to the active site and is presumed to not influence the active site, but it could influence the other nitrilase subunit.

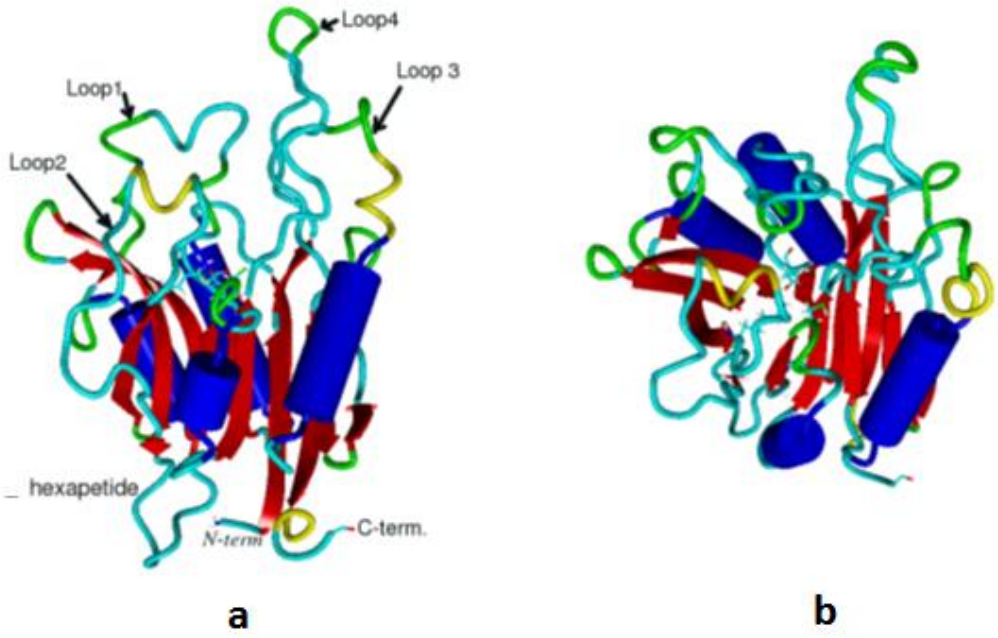


Figure 3.10: Model of nitrilase from *Neurospora crassa* obtained by Modeller: side view (a), top view (b) (author: Dr. N. Kulik, Department of Structure and Function of Proteins, Institute of Nanobiology and Structural Biology, AS CR)

The nitrilases from *A. niger* and *N. crassa* were studied by sedimentation velocity analysis. Both nitrilases formed a mixture of oligomers in the solution. *N. crassa* nitrilase formed a mixture of particles with sedimentation coefficients in the range 3 to 12S (which is equivalent to a monomer to hexa-/octamer) (Figure 3.11). The nitrilase from *A. niger* formed a more diverse mixture – particles with a sedimentation coefficient of up to 19S (which is equivalent to a hexadecamer) (Figure 3.12) (The sedimentation velocity analysis was made by Dr. O. Vaněk, Faculty of Science, Charles University in Prague).

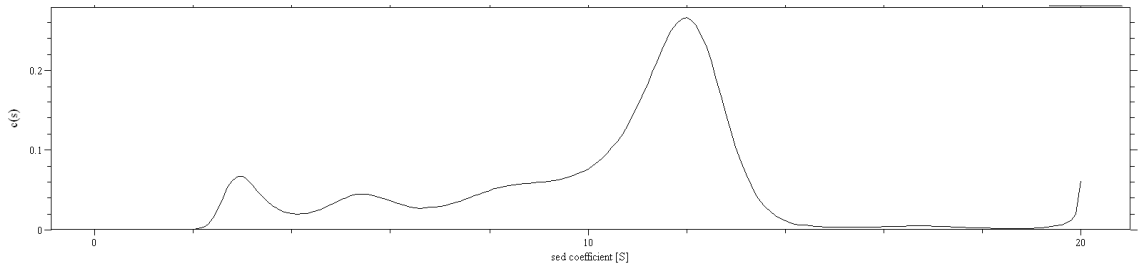


Figure 3.11: Sedimentation velocity analysis of nitrilase from *N. crassa* (author: Dr. O. Vaněk, Department of Biochemistry, Faculty of Science, Charles University in Prague)

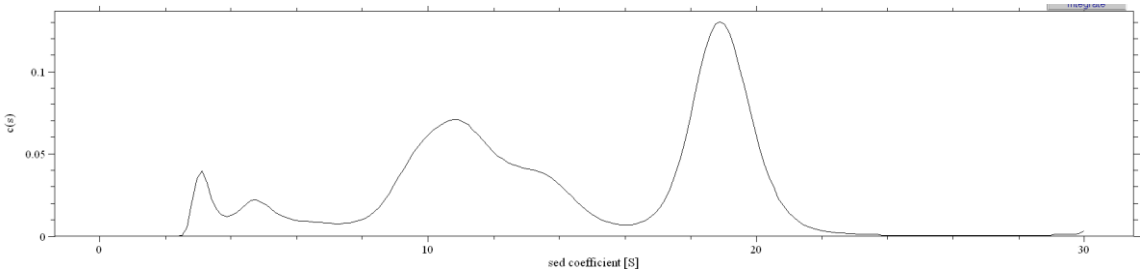


Figure 3.12: Sedimentation velocity analysis of nitrilase from *A. niger* (author: Dr. O. Vaněk, Department of Biochemistry, Faculty of Science, Charles University in Prague)

Due to the undefined mixture of oligomers obtained, none of the enzymes were suitable for crystallization.

3.6 Comparison of expression systems *E. coli* BL21 Gold (DE3) and *E. coli* JM109

Initially, expression was performed in *E. coli* BL21 Gold (DE3) (Agilent Technologies – Stratagene Products). In this host/vector system the nitrilase genes were ligated in the expression plasmid pET30a(+). Strains were grown in LB broth with kanamycin (100 µg/ml) at 30 °C for 3 h, nitrilase expression was induced with IPTG (0.5 mM) and the cultivation continued for 18 h.

Alternatively, expression was performed in *E. coli* JM109 (Viera and Messing, 1982) with nitrilase genes, containing His_6-tag, ligated in the vector pJOE2775. Strains were grown in LB broth with ampicillin (100 µg/ml) at 30 °C, nitrilase expression was induced by the addition of rhamnose (0.2 %) and cells cultivated for 18 hours.

The recombinant protein production was significantly slower with rhamnose induction (Figure 3.13). In contrast, under these conditions the specific activity was higher. Hence, *E. coli* JM109 cells were then chosen for the mutagenesis experiments (Table 3.4) (These experiments were done as a part of my three-month stay at the Institute of Microbiology, University of Stuttgart, Germany).

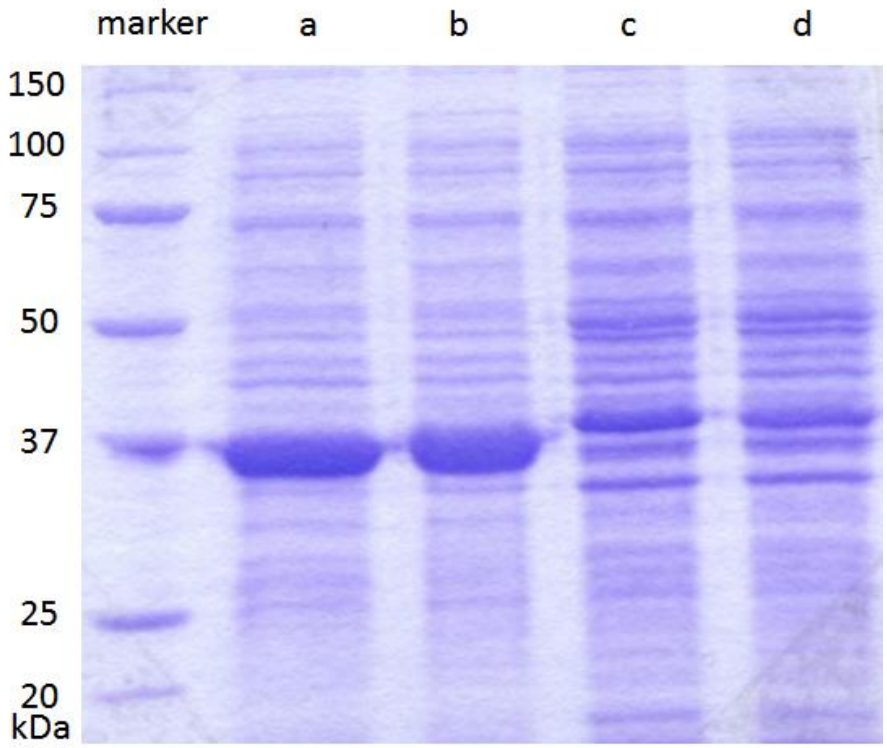


Figure 3.13: Comparison of nitrilases expressed by two different expression systems; *E. coli* BL21 with IPTG induction – *A. niger* and *N. crassa* (a, b), *E. coli* JM109 with rhamnose induction – *A. niger* and *N. crassa* (c, d)

Table 3.4: Conversion of (*R,S*)-mandelonitrile by resting cells of *E. coli* strains expressing nitrilases from *Neurospora crassa* (NitNc) and *Aspergillus niger* (NitAn) using various expression systems

Strain	Inducer	Specific activity (U/mg of protein) mandelic acid	Specific activity (U/mg of protein) mandelamide
E. coli BL21 (NitAn)	IPTG	0.9	-
E. coli BL21 (NitNc)	IPTG	0.4	-
E. coli JM109 (NitAn)	Rhamnose	2.3	-
E. coli JM109 (NitNc)	Rhamnose	3.1	1.5

In the Laboratory of Biotransformation, a similar comparative experiment was conducted. Slightly different conditions were used for nitrilase expression by *E. coli* BL21 Gold (DE3) (See section 3.4 for more details), but the results obtained were similar. The specific activity of nitrilase producing cells was higher for *E. coli* JM109 (data not shown).

Because of unsuccesful attempts at purifying the nitrilase variants expressed by the *E. coli* JM109 expression system, nitrilases expressed by *E. coli* BL21 Gold (DE3) were then used for further experiments.

3.7 Mutagenesis

A tryptophan residue is the most frequent amino acid residue next to the catalytic cysteine (toward the C terminus) in most nitrilases (Kiziak *et al.*, 2007; O'Reilly and Turner, 2003; Thuku *et al.*, 2009). Some nitrilases carry an alanine residue in the corresponding position, such as the nitrilase from *Klebsiella pneumoniae* ssp. *ozaenae* or *Pseudomonas fluorescens* EBC191 (Stalker *et al.*, 1988; Kiziak *et al.*, 2007). The histidine residue, the third amino acid from the cysteine (towards the C terminus) is also highly conserved in most nitrilases. An asparagine residue in the corresponding position can be found in *Aspergillus niger* K10 or in cyanide hydratases (Kaplan *et al.*, 2006b; O'Reilly and Turner, 2003; Thuku *et al.*, 2009).

Extensive site-directed mutagenesis experiments had been previously performed in the laboratory of a collaborative group (from the Institute of Microbiology, Stuttgart) with the nitrilase from *Pseudomonas fluorescens* EBC191. For this nitrilase, some point mutations close to the active site had been identified, which influenced the catalytic properties of the enzyme (Kiziak and Stolz, 2009; Sosedov *et al.*, 2010), e.g. resulting in a shift in the major products from acids to amides.

The following mutagenesis experiments were performed during my three-month stay at the Institute of Microbiology, University of Stuttgart (for more details, see Appendix 3).

The conversion of (*R,S*)-mandelonitrile and (*R,S*)-2-phenylpropionitrile by wild-type whole cell catalysts at pH 5 was analysed. The wild type nitrilase from *N. crassa* (NitNc) formed about 60 % mandelic acid and 40 % mandelamide from (*R,S*)-mandelonitrile. In contrast, the nitrilase from *A. niger* (NitAn) gave (*R*)-mandelic acid (e.e. = 93 %) from (*R,S*)-mandelonitrile and almost no amide. Both enzymes converted (*R,S*)-2-phenylpropionitrile stoichiometrically to (*R*)-2-phenylpropionic acid (e.e. = 90 and 35 % for *A. niger* and *N. crassa* nitrilase, respectively) and did not form any amide from this substrate.

Both wild-type nitrilases demonstrated a high degree of enantioselectivity for the formation of (*R*)-mandelic acid (e.e. > 90 %). The nitrilase from *N. crassa* also demonstrated a high degree of enantioselectivity for the formation of (*S*)-mandelamide (e.e. = 97 %).

In the nitrilases from *A. niger* and *N. crassa,* the tryptophan residue at position 163 and 168, respectively, and the histidine residue at position 170 and 175, respectively, were replaced (Figure 3.14). Trp residue and His residue were replaced with an Ala residue and Asn residue, respectively.

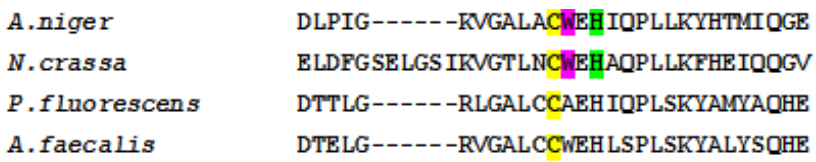


Figure 3.14: Sequences flanking catalytic cystein: C = catalytic cystein, W, H = replaced amino acids

The catalytic properties of the nitrilase variants were compared to the corresponding wild-type enzymes.

The replacement of Trp- with Ala-residues at position 163 and 168 in the nitrilase from *A. niger* and *N. crassa*, respectively, resulted in a pronounced increase in amide formation and decreased enzyme activity. The nitrilase variants from *A. niger* and *N. crassa* formed up to 33 % and 85 % of (*S*)-mandelamide (e.e. = 99 and 32 % for *A. niger* and *N. crassa* nitrilase, respectively) from (*R,S*)-mandelonitrile, respectively. The NitAn-W163A and NitNc-W168A variants demonstrated a lower degree of enantioselectivity for the formation of (*R*)-mandelic acid (Table 3.5, Table 3.6).

Table 3.5: Conversion of (*R,S*)-mandelonitrile by resting cells of *E. coli* JM109 expressing various variants of nitrilases from *Aspergillus niger.* MA = mandelic acid, MAA = mandelamide
[a]preferentially produced enantiomer, at about 30% conversion

Nitrilase variant	An	AnW163A	AnH165N
Activity (μmol_{MA} min^{-1} $mg^{-1}_{protein}$)	2.324	0.135	1.226
Activity (μmol_{MAA} min^{-1} $mg^{-1}_{protein}$)	-	0.067	0.021
MAA (% in total product)	-	33	10
e.e. MA (%)[a]	93 (R)	18 (R)	94 (R)
e.e. MAA (%)[a]	-	99 (S)	70 (S)

Table 3.6: Conversion of (*R,S*)-mandelonitrile by resting cells of *E. coli* JM109 expressing various variants of nitrilases from *Neurospora crassa.* MA = mandelic acid, MAA = mandelamide
[a]preferentially produced enantiomer, at about 30% conversion
[a]90% conversion (production of amide is slower than production of acid)

Nitrilase variant	Nc	NcW168A	NcH170N
Activity (μmol_{MA} min^{-1} $mg^{-1}_{protein}$)	3.117	0.103	0.443
Activity (μmol_{MAA} min^{-1} $mg^{-1}_{protein}$)	1.536	0.771	0.017
MAA (% in total product)	40	85	16
e.e. MA (%)[a]	91 (R)	78 (R)	94 (R)
e.e. MAA (%)[a]	97 (S)[b]	32 (S)	53 (S)[b]

The NitNc-W168N variant also formed a significant amount of (*S*)-2-phenylpropionamide (e.e. = 90 %) from (*R,S*)-2-phenylpropionitrile (Table 3.7,

Table 3.8). The specific activity of this variant with (*R,S*)-2-phenylpropionitrile was lower than that observed for the wild type nitrilase. Furthermore, a change in the preferentially formed enantiomer from (*R*)- to (*S*)-2-phenylpropionic acid was observed for both Trp- and Ala- mutants. The NitNc-W168A variant was highly enantioselective for the formation of (*S*)-2-phenylpropionic acid (e.e. = 98 %) and (*S*)-2-phenylpropionic acid amide (e.e. = 90 %).

Table 3.7: Conversion of (*R,S*)-2-phenylpropionitrile by resting cells of *E. coli* JM109 expressing various variants of nitrilases from *Aspergillus niger.* PPA = 2-phenylpropionic acid, PPAA = 2-phenylpropionamide
[a]preferentially produced enantiomer, at about 30% conversion

Nitrilase variant	An	AnW163A	AnH165N
Activity ($\mu mol_{PPA}\ min^{-1}\ mg^{-1}_{protein}$)	0.150	0.018	0.048
Activity ($\mu mol_{PPAA}\ min^{-1}\ mg^{-1}_{protein}$)	-	-	-
PPAA (% in total product)	-	-	-
e.e. PPA (%)[a]	90 (R)	63 (S)	94 (R)
e.e. PPAA (%)	-	-	-

Table 3.8: Conversion of (*R,S*)-2-phenylpropionitrile by resting cells of *E. coli* JM109 expressing various variants of nitrilases from *Neurospora crassa.* PPA = 2-phenylpropionic acid, PPAA = 2-phenylpropionamide
[a]preferentially produced enantiomer, at about 30% conversion

Nitrilase variant	Nc	NcW168A	NcH170N
Activity ($\mu mol_{PPA}\ min^{-1}\ mg^{-1}_{protein}$)	0.033	0.031	0.012
Activity ($\mu mol_{PPAA}\ min^{-1}\ mg^{-1}_{protein}$)	-	0.011	-
PPAA (% in total product)	-	31	-
e.e. PPA (%)[a]	35 (R)	98 (S)	18 (R)
e.e. PPAA (%)[a]	-	90 (S)	-

The nitrilase from *Pseudomonas fluorescens* EBC191 which carries an Ala-residue in position 165 (corresponding to positions 163 and 168 in the nitrilases from *A. niger* and *N. crassa*) exhibited a lower enantioselectivity for the formation of (*R*)-mandelic acid (e.e. = 31 %) compared to the wild-type nitrilases from *A. niger* and *N. crassa* (e.e. > 90 %). The importance of this amino acid position for the enantioselectivity of nitrilases was confirmed by the NitAn-W163A nitrilase variant generated here, which demonstrated a similar low degree of enantioselectivity for the formation of (*R*)-mandelic acid to the wild-type nitrilase from *Pseudomonas fluorescens* EBC191.

The replacement of His- with Asn-residues in positions 165 and 170 in the nitrilases from *A. niger* and *N. crassa,* respectively, led to decreased enzyme activities. Furthermore, an increased amide formation was observed for the nitrilase from *A. niger.* In contrast, the nitrilase from *N. crassa* formed less amides from (*R,S*)-mandelonitrile and (*R,S*)-2-phenylpropionitrile. No significant changes were observed in the enantiocomposition of the mandelic acid formed from (*R,S*)-mandelonitrile.

In many arylacetonitrilase sequences an insert consisting of six amino acids is present in the proximity of the catalytic cysteine residue. Deletion of this hexapeptide in the *N. crassa* nitrilase led to a complete loss of activity for phenylacetonitrile, despite the presumed distance from the active site in the tertiary structure, determined by homology modelling.

3.8 Conversion of high concentrations of (*R,S*)-mandelonitrile

(*R*)-Mandelic acid is a precursor of several antibiotics and serves as a general resolving agent (Zhang *et al.*, 2011). There is therefore interest in finding an enzyme able to hydrolyze (*R,S*)-mandelonitrile enantioselectively at higher rates.

Nitriles (especially mandelonitrile) can be toxic for the nitrilase at a higher concentration; thus the concentration of substrate is the limiting factor.

Most nitriles are poorly soluble in aqueous solution. A cosolvent can be added to improve the solubility of the nitrile; on the other hand, it can inhibit the enzyme activity. The cosolvent concentration must therefore be carefully considered (Chen *et al.*, 2009).

There are many nitrilases hydrolyzing (*R,S*)-mandelonitrile, such as the nitrilases from *Pseudomonas fluorescens, Burkholderia xenovorans* or *Bradyrhizobium japonicum*, but they are rarely used at higher substrate concentrations (Kiziak *et al.*, 2005; Seffernick *et al.*, 2009; Zhu *et al.*, 2007). Only the nitrilase from *Alcaligenes* sp. ECU0401 has been used to convert higher amount of mandelonitrile (300 – 500 mM), with toluene as cosolvent – added to protect the enzyme from inhibition by the substrate (Zhang *et al.*, 2011). No wild-type producer of an (*R,S*)-mandelonitrile degrading nitrilase is known in filamentous fungi.

Resting cells of *E. coli* BL 21 (DE3) Gold expressing *N. crassa* and *A. niger* were used for the hydrolysis of 500 mM (*R,S*)-mandelonitrile into (*R*)-mandelic acid. A biphasic system containing toluene was used for the reaction.

The reaction proceeded in 50-ml Falcon tubes, in a total volume of 10 ml. The toluene content was 4 or 10 %. Moderate shaking (350 rpm) was crucial for efficiency

of the reaction - mandelonitrile was only partially dissolved in the reaction mixture and the cells were in part protected from its toxic impact.

The inhibition of the enzyme by high concentrations of substrate was decreased by the addition of toluene at concentrations of 4 to 10 % of the total reaction volume. On the other hand, a higher concentration of toluene (10 % vs 4 %) decreased the 500 mM mandelonitrile conversion to only 50 %.

An increase in pH from 8 to 9 led to an increase in e.e. of (*R*)-mandelic acid for both enzymes (80 to 96 % and 84 to 92 % for *N. crassa* and *A. niger*, respectively) and decrease in amide formation (30 to 7 % and 1.5 to 0.8 % for *N. crassa* and *A. niger*, respectively). The enantiomeric excess was measured after 300 min of reaction when an almost full conversion of substrate to product occured. A further increase in pH to 10 led to a decrease in nitrilase activity, an 80 % conversion of 500 mM mandelonitrile being achieved. Amide formation and e.e. of products was comparable to the reaction at pH 9.

The optimal conditions were determined as pH 9; 4 % toluene and moderate shaking (350 rpm). Mandelonitrile was converted almost completely, reaching 94 and 97 % conversion, using the enzymes from *A. niger* and *N. crassa*, respectively (under optimized conditions) (Figure 3.15, Figure 3.16).

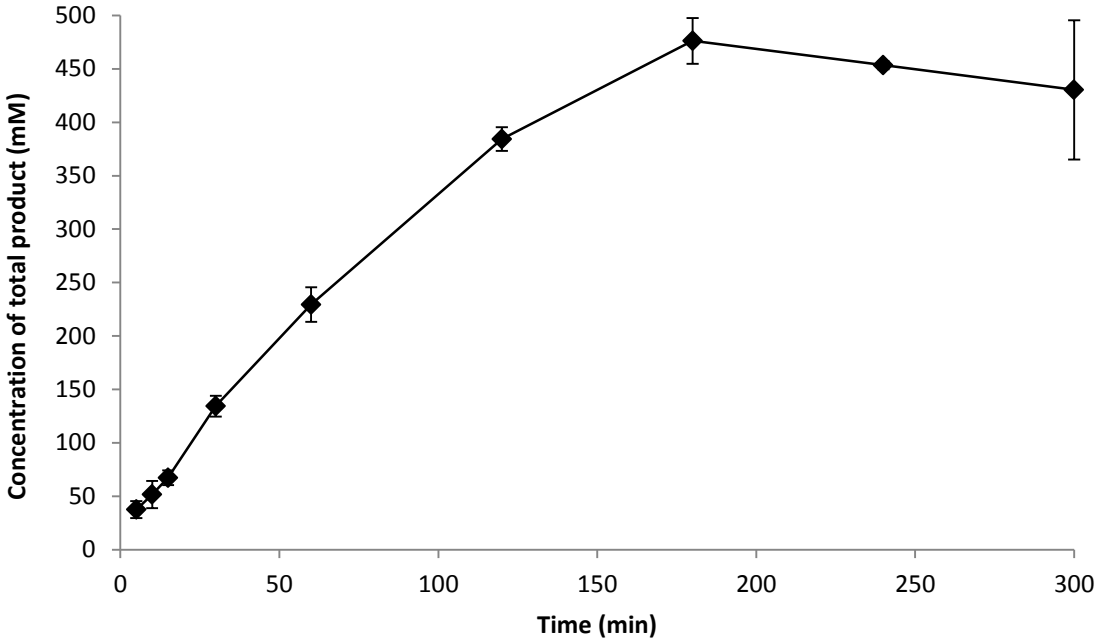


Figure 3.15: Conversion of 500 mM (*R,S*)-mandelonitrile by nitrilase from *A. niger* under optimized conditions (pH 9; 4 % of toluene). Total product = mandelic acid (99.2 %) + mandelamide (0.08 %)

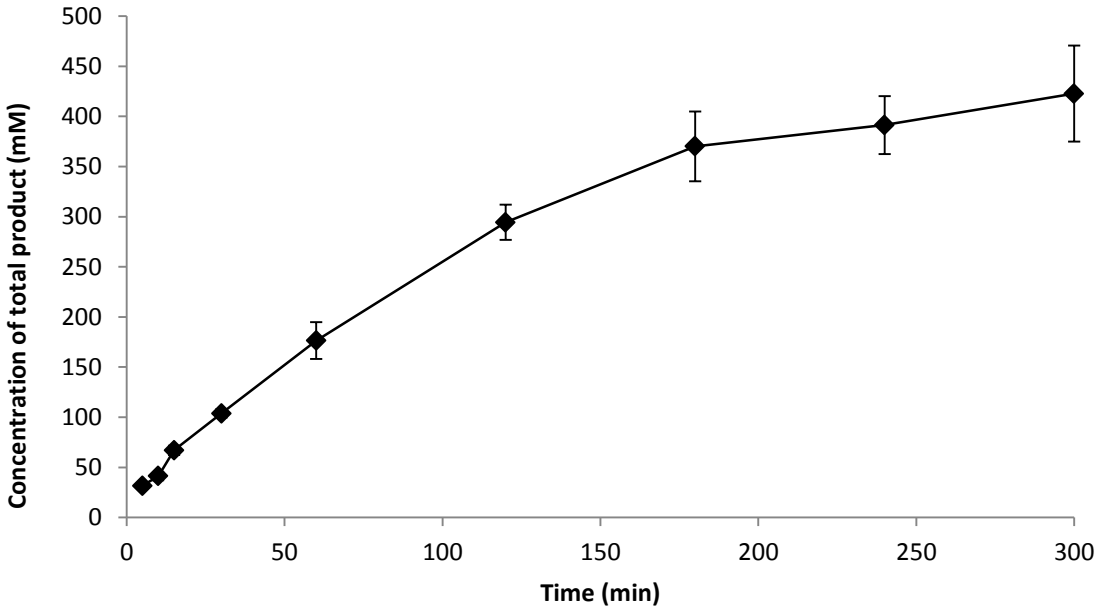


Figure 3.16: Conversion of 500 mM (*R,S*)-mandelonitrile by nitrilase from *N. crassa* under optimized conditions (pH 9; 4 % of toluene). Total product = mandelic acid (93 %) + mandelamide (7 %)

(*S*)-Mandelonitrile, which is not a good substrate for the nitrilase, undergoes a rapid racemization under these conditions. Therefore, a high yield of (*R*)-mandelic acid is obtained.

The catalyst productivities of these fungal nitrilases were higher than in the bacterial nitrilases that have been studied so far. With the *N. crassa* and *A. niger* nitrilase, 3.8 and 4.8 g mandelic acid per gram of wet cell weight was obtained, respectively, compared to 0.45 g mandelic acid per gram of wet cell weight obtained with the *Alcaligenes faecalis* ATCC 8750 nitrilase or 0.39 g mandelic acid per gram of immobilized cell obtained with the *Pseudomonas putida* MTCC 5110 nitrilase in the form of alginate-trapped cells (Yamamoto *et al.*, 1991; Banerjee *et al.*, 2006). Thus the use of these enzymes in the production of high amounts of optically pure α-hydroxycarboxylic acid is promising.

4 Conclusions

The first aim of this work was to find sequences encoding putative nitrilases in GenBank and to express them in suitable hosts. The *E. coli* expression system was tested and seemed to be suitable for the production of fully active recombinant fungal nitrilases. Among others, two novel fungal arylacetonitrilases from *Aspergillus niger* CBS 513.88 and *Neurospora crassa* OR74A, whose sequences were obtained by genome mining, were succesfully expressed in *E. coli* in high levels (23 000 and 69 000 units per liter, respectively).

The catalytic properties of the nitrilases from the respective native producers were also examined. The production of the enzyme was very low compared to the production of recombinant nitrilases in *E. coli.* Therefore, the recombinant nitrilases were chosen for further experiments.

Both recombinant fungal nitrilases were purified and characterized. The optimal pH and temperature were determined as 9 and 38 °C, respectively, for both nitrilases and both enzymes were stable at temperatures below 35 °C. Both nitrilases were defined as arylacetonitrilases with an activity for phenylacetonitrile and other arylaliphatic nitriles.

The effect of co-expression with a set of molecular chaperones was tested. Only a slight increase in nitrilase activity was observed after the co-expression of the nitrilases with the chaperonine system GroEL/GroES and the trigger factor (by a factor of 1.25).

The structural properties of the nitrilases from *A. niger* and *N. crassa* were examined with various methods, such as electron microscopy, homology modelling or sedimentation velocity analysis. None of these enzymes were suitable for crystallization, due to the formation of an undefined mixture of oligomers, observed by analytical ultracentrifugation.

Selected amino acid residues close to the catalytic cysteine residue – tryptophan or histidine – were replaced by alanine and asparagine, in order to compare the catalytic properties of these variants to the respective wild-type nitrilases.

The nitrilase from *N. crassa* produced high amounts of (*S*)-mandelamide (40 % of total product) at pH 5, at which the decomposition of (*R,S*)-mandelonitrile is low. The amount of amide strongly increased in the reaction with the W168A variant of the *N. crassa* nitrilase, up to 85 % of the total product. The activity and enantioselectivity of all the nitrilase variants were lower than the wild-type ones. Moreover, during the conversion of (*R,S*)-2-phenylpropionitrile, a change in the preferentially formed enantiomer from (*R*)- to (*S*)-2-phenylpropionic acid was observed for both the W163A and W168A mutants.

Certain amino acids close to the catalytic centre of nitrilase have similar effects on enantioselectivity and chemoselectivity in distantly related nitrilases. This is probably because the tertiary structures of nitrilases are generally conserved, though they often share only a low degree of amino acid identity. This fact can be used to predict the catalytic properties of nitrilases, whose sequences can be found in databases, or for the site-specific mutagenesis of desired amino acids.

Up to 500 mM (*R,S*)-mandelonitrile was converted by the nitrilases. These nitrilases were able to convert (*R,S*)-mandelonitrile in high concentrations even without the addition of toluene as cosolvent. On the other hand, the presence of cosolvent positively influenced e.e. of produced (*R*)-mandelic acid for both nitrilases (e.e. increased from 69 to 92 % for *A. niger* nitrilase. Additionaly, the cosolvent also decreased the amount of the by-product formed (the respective amide). An increase in buffer pH had a similar effect.

New fungal arylacetonitrilases have potential for the synthesis of optically pure α-substituted carboxylic acids or amides from racemic substrates. According to the relationship between primary structure and enzyme catalytic properties, new genes encoding nitrilases can be found in databases, expressed and used for specific needs, such as the manufacture of pharmaceuticals or bioremediation.

5 References

Alonso, F. O. M., Oestricher, E. G., Antunes, O. A. C.: Production of enantiomerically pure D-phenylglycine using *Pseudomonas aeruginosa* 10145 as biocatalyst. *Braz J Chem Eng* **25**, 1-8 (2008)

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J.: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402 (1997)

Asano, Y.: Overview of screening for new microbial catalysts and their uses in organic synthesis - selection and optimization of biocatalysts. *J Biotechnol* **94**, 705-708 (2002)

Banerjee, A., Sharma, R., Banerjee, U. C.: The nitrile-degrading enzymes: current status and future prospects. *Appl Microbiol Biotechnol* **60**, 33-44 (2002)

Banerjee, A., Kaul, P., Banerjee, U. C.: Enhancing the catalytic potential of nitrilase from *Pseudomonas putida* for stereoselective nitrile hydrolysis. *Appl Microbiol Biotechnol* **72**, 77-87 (2006)

Banerjee, A., Dubey, S., Kaul, P., Barse, B., Piotrowski, M., Banerjee, U. C.: Enantioselective nitrilase from *Pseudomonas putida*: Cloning, heterologous expression, and bioreactor studies. *Mol Biotechnol* **41**, 35-41 (2009)

Basile, L. J., Wilson, R., Sewell, B. T., Benedik, M. J.: Genome mining of cyanide-degrading nitrilases from filamentous fungi. *Appl Microbiol Biotechnol* **80**, 427-435 (2008)

Bengis-Garber, C., Gutman, A. L.: Selective hydrolysis of dinitriles into cyano-carboxylic acids by *Rhodococcus rhodochrous* N.C.I.B. 11216. *Appl Microbiol Biotechnol* **32**, 11-16 (1989)

Bhalla, T. C., Miura, A., Wakamoto, Y., Furuhashi, K.: Asymmetric hydrolysis of α-aminonitriles to optically active amino acids by a nitrilase of *Rhodococcus rhodochrous* PA-34. *Appl Microbiol Biotech* **37**, 184-190 (1992)

Bhalla, T. C., Sharma, M., Sharma, N. N.: Nitrile metabolizing yeasts. In *Yeast Biotechnology: Diversity and Applications.* Edited by Satyanarayana, T., Kunze, G., Netherlands: Springer, 715-735 (2009)

Bjarnholt, N., Møller, B. L.: Hydroxynitrile glucosides. *Phytochemistry* **69**, 1947-1961 (2008)

Bork, P., Koonin, E. V.: A new family of carbon-nitrogen hydrolases. *Protein Sci* **3**, 1344-1346 (1994)

Brady, D., Beeton, A., Zeevaart, J., Kgaje, C., van Rantwijk, F., Sheldon, R. A.: Characterization of nitrilase and nitrile hydratase biocatalytic systems. *Appl Microbiol Biotechnol* **64**, 76-85 (2004)

Brenner, C.: Catalysis in the nitrilase superfamily. *Curr Opin Struc Biol* **12**, 775-782 (2002)

Brewis, E. A., Walt, J. P. D. V., Prior, B. A.: The utilization of aromatic, cyclic and heterocyclic nitriles by yeasts. *Syst Appl Microbiol* **18**, 338-342 (1995)

Chen, J., Zheng, R.-C., Zheng, Y.-G., Shen, Y.-C.: Microbial transformation of nitriles to high-value acids or amides. *Adv Biochem Engin/Biotechnol* **113**, 33-77 (2009)

Chmura, A., Shapovalova, A. A., van Pelt, S., van Rantwijk, F., Tourova, T. P., Muyzer, G., Sorokin, D. Y.: Utilization of arylaliphatic nitriles by haloalkaliphilic *Halomonas nitrilicus* sp. nov. isolated from soda soils. *Appl Microbiol Biotechnol* **81**, 371-378 (2008)

Cluness, M. J., Turner, P. D., Clements, E., Brown, D. T., O'Reilly, C.: Purification and properties of cyanide hydratase from *Fusarium lateritium* and analysis of the corresponding *chy*1 gene. *J Gen Microbiol* **139**, 1807-1815 (1993)

Conn, E.E.: Biosynthesis of cyanogenic glycosides. *Naturwissenschaften* **66**, 28-34 (1979)

Fernandes, B. C. M., Mateo, C., Kiziak, C., Chmura, A., Wacker, J., Rantwijk, F., Stolz, A., Sheldon, R. A.: Nitrile hydratase activity of a recombinant nitrilase. *Adv Synth Catal* **348**, 2597-2603 (2006)

Fournand, D., Bigey, F., Arnaud, A.: Acyl transfer activity of an amidase from *Rhodococcus* sp. strain R312: formation of a wide range of hydroxamic acids. *Appl Environ Microbiol* **64**, 2844-2852 (1998)

Fry, W. E., Millar, R. L.: Cyanide degradation by an enzyme from *Stemphylium loti*. *Arch Biochem Biophys* **151**, 468-474 (1972)

Godtfredsen, S. E., Ingvorsen, K., Yde, B., Anderson, O.: In *Biocatalysis in organic synthesis*. Edited by Tramper, J., Vanderplas, H. C., Linko, P., Netherlands: Elsevier, 3-18 (1985)

Goldlust, A., Bohak, Z.: Induction, purification, and characterization of the nitrilase of *Fusarium oxysporum* f. sp. *melonis*. *Biotechnol Appl Biochem* **11**, 581-601 (1989)

Gong, J.-S., Lu, Z.-M., Li, H., Shi, J.-S., Zhou, Z.-M., Xu, Z.-H.: Nitrilases in nitrile biocatalysis: Recent progress and forthcoming research. *Microbial Cell factories* **11** (2012)

Gradley, M. L., Deverson, C. J. F., Knowles, C. J.: Asymmetric hydrolysis of (*R*)-(–), (*S*)-(+)-2-methylbutyronitrile by *Rhodococcus rhodochrous* NCIMB 11216. *Arch Microbiol* **161**, 246-251 (1994)

Gupta, N., Balomajumder, C., Agarwal, V. K.: Enzymatic mechanism and biochemistry for cyanide degradation: A review. *J Hazard Mater* **176**, 1-13 (2010)

Hardy, R. W. F., Burns, R. C., Hebert, R. R., Holsten, R. D., Jackson, E. K.: Biological nitrogen fixation: A key to world protein. *Plant Soil* **35***,* 561-590 (1971)

Harper, D. B.: Fungal degradation of aromatic nitriles. *Biochem J* **167**, 685-692 (1977)

Heinemann, U., Engels, D., Bürger, S., Kiziak, C., Mattes, R., Stolz, A.: Cloning of a nitrilase gene from the cyanobacterium *Synechocystis* sp. strain PCC6803 and heterologous expression and characterization of the encoded protein. *Appl Environ Microbiol* **69**, 4359-4366 (2003)

Ingvorsen, K., Højer-Pedersen, B., Godtfredsen, S. E.: Novel cyanide-hydrolyzing enzyme from *Alcaligenes xylosoxidans* subsp. *denitrificans. Appl Environ Microbiol* **57**, 1783-1789 (1991)

Jandhyala, D., Berman, M., Meyers, P. R., Sewell, B. T., Wilson, R. C., Benedik, M. J.: CynD, the cyanide dihydratase from *Bacillus pumilus*: Gene cloning and structural studies. *Appl Environ Microbiol* **69**, 4794-4805 (2003)

Janowitz, T., Trompetter, I., Piotrowski, M.: Evolution of nitrilases in glucosinolate-containing plants. *Phytochemistry* **70**, 1680-1686 (2009)

Kaczanowski, S., Zielenkiewicz, P.: Why similar protein sequences encode similar three-dimensional structures? *Theor Chem Acc* **125**, 643–650 (2010)

Kaplan, O., Vejvoda, V., Charvátová-Pišvejcová, A., Martínková, L.: Hyperinduction of nitrilases in filamentous fungi. *J Ind Microbiol Biotechnol* **33**, 891-896 (2006a)

Kaplan, O., Vejvoda, V., Plíhal, O., Pompach, P., Kavan, D., Bojarová, P., Bezouška, K., Macková, M., Cantarella, M., Jirků, V., Křen, V., Martínková, L.: Purification and characterization of a nitrilase from *Aspergillus niger* K10. *Appl Microbiol Biotechnol* **73**, 567-575 (2006b)

Kaplan, O., Bezouška, K., Malandra, A., Veselá, A. B., Petříčková, A., Felsberg, J., Rinágelová, A., Křen, V., Martínková, L.: Genome mining for the discovery of new nitrilases in filamentous fungi. *Biotechnol Lett* **33**, 309-312 (2011)

Kaplan, O., Veselá, A. B., Petříčková, A., Pasquarelli, F., Pičmanová, M., Rinágelová, A., Bhalla, T. C., Pátek, M., Martínková, L.: A comparative study of nitrilases identified by genome mining. *Mol Biotechnol* **54***,* 996-1003 (2013)

Kaul, P., Banerjee, A., Mayilraj, S., Banerjee, U. C.: Screening for enantioselective nitrilases: Kinetic resolution of racemic mandelonitrile to (*R*)-(–)-mandelic acid by new bacterial isolates. *Tetrahedron: Asymmetry* **15**, 207-211 (2004)

Kiziak, C., Conradt, D., Stolz, A., Mattes, R., Klein, J.: Nitrilase from *Pseudomonas fluorescens* EBC191: cloning and heterologous expression of the gene and biochemical charactrerization of the recombinant enzyme. *Microbiology* **151**, 3639-3648 (2005)

Kiziak, C., Klein, J., Stolz, A.: Influence of different carboxy-terminal mutations on the substrate-, reaction- and enantiospecificity of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191. *Protein Eng Des Sel* **8**, 385-396 (2007)

Kiziak, C., Stolz, A.: Identification of amino acid residues responsible for the enantioselectivity and amide formation capacity of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191. *Appl Environ Microbiol* **75**, 5592-5599 (2009)

Kobayashi, M., Nagasawa, T., Yamada, H.: Regiospecific hydrolysis of dinitrile compounds by nitrilase from *Rhodococcus rhodochrous* J1. *Appl Microbiol Biotechnol* **29**, 231-233 (1988)

Kobayashi, M., Nagasawa, T., Yamada, H.: Nitrilase of *Rhodococcus rhodochrous* J1: Purification and characterization. *Eur J Biochem* **182**, 349-356 (1989)

Kobayashi, M., Yanaka, M., Nagasawa, T., Yamada, H.: Purification and characterization of a novel nitrilase of *Rhodococcus rhodochrous* K22 that acts on aliphatic nitriles. *J Bacteriol* **172**, 4807-4815 (1990)

Kobayashi, M., Komeda, H., Yanaka, N., Nagasawa, T., Yamada, H.: Nitrilase from *Rhodococcus rhodochrous* J1. Sequencing and overexpression of the gene and identification of an essential cysteine residue. *J Biol Chem* **267**, 20746-20751 (1992)

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

Layh, N., Parratt, J., Willetts, A.: Characterization and partial purification of an enantioselective arylacetonitrilase from *Pseudomonas fluorescens* DSM 7155. *J Mol Catal B: Enzym* **5**, 467-474 (1998)

Legras, J. L., Chuzel, G., Arnaud, A., Galzy, P.: Natural nitriles and their metabolism. *World J Microb Biot* **6**, 83-108 (1990)

Lévy-Schil, S., Soubriere, F., Crutz-Le Coq, A. M., Faucher, D., Crouzet, J., Pétré, D.: Aliphatic nitrilase from a soil-isolated *Comamonas testosteroni* sp.: gene cloning and overexpression, purification and primary structure. *Gene* **161**, 15-20 (1995)

Martínková, L., Vejvoda, V., Křen, V.: Selection and screening for enzymes of nitrile metabolism. *J Biotechnol* **133**, 318-326 (2008)

Martínková, L., Vejvoda, V., Kaplan, O., Kubáč, D., Malandra, A., Cantarella, M., Bezouška, K., Křen, V.: Fungal nitrilases as biocatalysts: Recent developments. *Biotechnol Adv* **27**, 661-670 (2009)

Martínková, L., Křen, V.: Biotransformation with nitrilases. *Curr Opin Chem Biol* **14**, 130-137 (2010)

Mateo, C., Palomo, J. M., van Langen, L. M., van Rantwijk, F., Sheldon, R. A.: A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates. *Biotechnol Bioeng* **86**, 273-276 (2004)

Matthew, C., Nagasawa, T., Kobayashi, M., Yamada, H.: Nitrilase catalyzed production of nicotinic acid from 3-cyanopyridine in *Rhodococcus rhodochrous* J1. *Appl Environ Microbiol* **54**, 1030-1032 (1988)

McCluskey, K.: The fungal genetics stock center: From molds to molecules. *Adv Appl Microbiol* **52**, 246-262 (2003)

Meyers, P. R., Rawlings, D. E., Woods, D. R., Lindsey, G. G.: Isolation and characterization of a cyanide dihydratase from *Bacillus pumilus* C1. *J Bacteriol* **175**, 6105-6112 (1993)

Miller, J. M., Conn, E.E.: Metabolism of hydrogen cyanide by higher plants. *Physiol Plant* **65**, 1199-1202 (1980)

Mueller, P., Egarova, K., Vorgias, E. C., Boutou, E., Trauthwein, H., Verseck, S., Antranikian, G.: Cloning, expression and characterization of a thermoactive nitrilase from the hyperthermophilic archaeon *Pyrococcus abyssi*. *Protein Expression Purif* **47**, 672-681 (2006)

Nagasawa, T., Kobayashi, M., Yamada, H.: Optimum culture conditions for the production of benzonitrilase by *Rhodococcus rhodochrous* J1. *Arch Microbiol* **150**, 89-94 (1988)

Nagasawa, T., Nakamura, T., Yamada, H.: ε-caprolactam, a new powerful inducer for the formation of *Rhodococcus rhodochrous* J1 nitrilase. *Arch Microbiol* **155**, 13-17 (1990)

Nakai, T., Hasegawa, T., Yamashita, E., Yamamoto, M., Kumasaka, T., Ueki, T., Nanba, H., Ikenaka, Y., Takahashi, S., Sato, M., Tsuhikara, T.: Crystal structure of *N*-carbamyl-D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases. *Structure* **8**, 729-739 (2000)

Nolan, L. M., Harnedy, P. A., Turner, P., Hearne, A. B., O'Reilly C.: The cyanide hydratase enzyme of *Fusarium lateritium* also has nitrilase activity. *FEMS Microbiol Lett* **221**, 161-165 (2003)

O'Reilly, C., Turner, P. D.: The nitrilase family of CN hydrolysing enzymes – a comparative study. *J Appl Microbiol* **95**, 1161-1174 (2003)

Osswald, S., Wajant, H., Effenberger, F.: Characterization and synthetic applications of recombinant AtNIT1 from *Arabidopsis thaliana. Eur J Biochem* **269**, 680-687 (2002)

Pace, H. C., Hodawadekar, S. C., Draganescu, A., Huang, J., Bieganowski, P., Pekarsky, Y., Croce, C. M., Brenner, C.: Crystal structure of the worm NitFhit Rosetta Stone protein reveals a Nit tetramer binding two Fhit dimers. *Curr Biol* **10**, 907-917 (2000)

Pace, H., C., Brenner, C.: The nitrilase superfamily: classification, structure and function. *Genome biology* **2** (2001)

Panova, A., Mersinger, L. J., Liu, Q., Foo, T., Roe, D. C., Spillan, W. L., Sigmund, A. E., Ben-Bassat, A., Wagner, L. W., O'Keefe, D. P., Wu, S., Petrillo, K. L., Payne, M. S., Breske, S. T., Gallagher, F. G., DiCosimo, R.: Chemoenzymatic synthesis of glycolic acid. *Adv Synth Catal* **349**, 1462-1474 (2007)

Petříčková, A., Veselá, A. B., Kaplan, O., Kubáč, D., Uhnáková, B., Malandra, A., Felsberg, J., Rinágelová, A., Weyrauch, P., Křen, V., Bezouška, K., Martínková, L.: Purification and characterization of heterologously expressed nitrilases from filamentous fungi. *Appl Microbiol Biotechnol* **93**, 1553-1561 (2012)

Piotrowski, M.: Primary or secondary? Versatile nitrilases in plant metabolism. *Phytochemistry* **69**, 2655-2667 (2008)

Prasad, S., Misra, A., Jangir, V., Awasthi, A., Raj, J., Bhalla T.: A propionitrile-induced nitrilase of *Rhodococcus* sp. NDB 1165 and its application in nicotinic acid synthesis. *World J Microbiol Biotechnol* **23**, 345-353 (2007)

Raczynska, J. E., Vorgias, C. E., Antranikian, G., Rypniewski, W.: Crystalographic analysis of a thermoactive nitrilase. *J Struct Biol* **173**, 294-302 (2011)

Robertson, D. E., Chaplin, J. A., DeSantis, G., Podar, M., Madden, M., Chi, E., Richardson, T., Milan, A., Miller, M., Weiner, D. P., Wong, K., McQuaid, J., Farwell, B., Preston, L. A., Tan, X., Snead, M. A., Keller, M., Mathur, E., Kretz, P., Burk, M. J., Short, J.

M.: Exploring nitrilase sequence space for enantioselective catalysis. *Appl Environ Microbiol* **70**, 2429-2436 (2004)

Seffernick, J. L., Samanta, S. K., Louie, T. M., Wackett, L. P., Subramanian, M.: Investigative mining of sequence data for novel enzymes: A case study with nitrilases. *J Biotechnol* **143**, 17-26 (2009)

Shen, M., Liu, Z.-Q., Zheng, Y.-G., Shen, Y.-C.: Enhancing endo-nitrilase production by a newly isolated *Arthrobacter nitroguajacolicus* ZJUTB06-99 through optimization of culture medium. *Biotechnol Bioproc Eng* **14**, 795-802 (2009)

Soriano-Maldonado, P., Martínez-Gomez, A. I., Andjújar-Sánchez, M., Neira, J. L., Clemente-Jiménez, J. M., Las Heras-Vásquez, F. J., Rodríguez-Vico, F., Martínez-Rodríguez, S.: Biochemical and mutational studies of the *Bacillus cereus* CECT 5050T formamidase support the existence of a C-E-E-K tetrad in several members of the nitrilase superfamily. *Appl Environ Microbiol* **77**, 5761-5769 (2011)

Sosedov, O., Matzer, K., Bürger, S., Kiziak, C., Baum, S., Altenbucher, J., Chmura, A., van Rantwijk, F., Stolz, A.: Construction of recombinant *Escherichia coli* catalysts which simultaneously express an (*S*)-oxynitrilase and different nitrilase variants for the synthesis of (*S*)-mandelic acid and (*S*)-mandelic amide from benzaldehyde and cyanide. *Adv Synth Catal* **351**, 1531-1538 (2009)

Sosedov, O., Baum, S., Matzer, K., Kiziak, C., Stolz, A.: Construction and application of variants of the *Pseudomonas fluorescens* EBC191 arylacetonitrilase for increased production of acids or amides. *Appl Environ Microbiol* **76**, 3668-3674 (2010)

Stalker, D. M., McBride, K. E.: Cloning and expression in *Escherichia coli* of a *Klebsiella ozaenae* plasmid-borne gene encoding a nitrilase specific for the herbicide bromoxynil. *J Bacteriol* **169**, 955-960 (1987)

Stalker, D. M., Malyj, L. D., McBride, K. E.: Purification and properties of a nitrilase specific for the herbicide bromoxynil and corresponding nucleotide sequence analysis of the bxn gene. *J Biol Chem* **263**, 6310-6314 (1988)

Šnajdrová, R., Kristová-Mylerová, V., Crestia, D., Nikolau, K., Kuzma, M., Lemaire, M., Galliene, E., Bolte, J., Bezouška, K., Křen, V., Martínková, L.: Nitrile biotransformation by *Aspergillus niger*. *J Mol Catal B-Enzym* **29**, 227-232 (2004)

Thimann, K., Mahadevan, S.: Nitrilase II, its substrate specificity and possible mode of action. *Arch Biochem Biophys* **107**, 62-68 (1964)

Thuku, R. N., Brady, S., Benedik, M. J., Sewell, B. T.: Microbial nitrilases: versatile, spiral forming, industrial enzymes. *J Appl Microbiol* **106**, 703-727 (2009)

Vejvoda, V., Kaplan, O., Klozová, J., Masák, J., Čejková, A., Jirků, V., Stloukal, R., Martínková, L.: Mild hydrolysis of nitriles by *Fusarium solani* strain O1. *Folia Microbiol* **51**, 251-256 (2006)

Vejvoda, V., Kaplan, O., Bezouška, K., Pompach, P., Šulc, M., Cantarella, M., Benada, O., Uhnáková, B., Rinágelová, A., Lutz-Wahl, S., Fischer, L., Křen, V., Martínková, L.: Purification and characterization of a nitrilases from *Fusarium solani* O1. *J Mol Catal B: Enzym* **50**, 99-106 (2008)

Vejvoda, V., Kubáč, D., Davidová, A., Kaplan, O., Šulc, M., Šveda, O., Chaloupková, R., Martínková, L.: Purification and characterization of nitrilase from *Fusarium solani* IMI196840. *Process Biochem* **45**, 1115-1120 (2010)

Veselá, A., Petříčková, A., Weyrauch, P., Martínková, L.: Heterologous expression, purification and characterization of arylacetonitrilases from *Nectria haematococca* and *Arthroderma benhamiae*. *Biocatal Biotransform* **31**, 49-56 (2013)

Vetter, J.: Plant cyanogenic glycosides. *Toxicon* **38**, 11-36 (2000)

Viera, J., Messing, J.: The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259-268 (1982)

Vorwerk, S., Biernacki, S., Hillerbrand, H., Janzik, I., Muller, A., Weiler, E. W., Piotrowski, M.: Enzymatic characterization of the recombinant *Arabidopsis thaliana* nitrilase subfamily encoded by the NIT2/NIT1/NIT3-gene cluster. *Planta* **212**, 508-516 (2001)

Watanabe, A., Yano, K., Ikebukuro, K., Karube, I.: Cloning and expression of a gene encoding cyanidase from *Pseudomonas stutzeri* AK61. *Appl Microbiol Biotechnol* **50**, 93-97 (1998)

Wittstock, U., Halkier, B. A.: Glucosinolate research in the *Arabidopsis* era. *Trends Plant Sci* **7**, 263-270 (2002)

Wu, S., Fogiel, A. J., Petrillo, K. L., Jackson, R. E., Parker, K. N., DiCosimo, R., Ben-Bassat, A., O'Keefe, D. P., Payne, M. S.: Protein engineering of nitrilase for chemoenzymatic production of glycolic acid. *Biotechnol Bioeng* **99**, 717-720 (2008)

Xue, Y.-P., Xu, S.-Z, Liu, Z.-Q., Zheng, Y.-G., Shen, Y.-C.: Enantioselective biocatalytic hydrolysis of (*R,S*)-mandelonitrile for production of (*R*)-(–)-mandelic acid by a newly isolated mutant strain. *J Ind Microbiol Biotechol* **38**, 337-345 (2011)

Yamada, H., Nagasawa, T.: Production of useful amides by enzymatic hydration of nitriles. *Ann N Y Acad Sci* **613**, 142-154 (1990)

Yamamoto, K., Ueno, Y., Otsubo, K., Kawakami, K., Komatsu, K.: Production of *S*-(+)-ibuprofen from a nitrile compound by *Acinetobacter* sp. strain AK226. *Appl Environ Microbiol* **56**, 3125-3129 (1990)

Yamamoto, K., Oishi, K., Fujimatsu, I., Komatsu, K.: Production of R-(–)-mandelic acid from mandelonitrile by *Alcaligenes faecalis* ATCC 8750. *Appl Environ Microbiol* **57**, 3028-3032 (1991)

Yanase, H., Sakamoto, A., Okamoto, K., Kita, K., Sato, Y.: Degradation of the metal-cyano complex tetracyanonickelate (II) by *Fusarium oxysporum* N-10. *Appl Microbiol Biotechnol* **53**, 328-334 (2000)

Zagrobelny, M., Bak, S., Vinther Rasmusen, A., Jørgensenm B., Naumann, C. N., Lindberg Møller, B.: Cyanogenic glucosides and plant-insect interactions. *Phytochemistry* **65**, 293-306 (2004)

Zhang, Z.-J., Xu, J.-H., He, Y.-C., Ouyang, L.-M., Liu, Y.-Y.: Cloning and biochemical properties of a highly thermostable and enantioselective nitrilase from *Alcaligenes* sp. ECU0401 and its potential for (*R*)-(–)-mandelic acid production. *Bioproc Biosyst Eng* **34**, 315-322 (2011)

Zheng, Y. G., Chen, J., Liu, Z. Q., Wu, M. H., Xing, L. Y., Shen, Y. C.: Isolation, identification and characterization of *Bacillus subtilis* ZJB-063, a versatile nitrile-converting bacterium. *Appl Microbiol Biotechnol* **77**, 985-993 (2008)

Zhu, C., Mukherjee, E. R., Biehl, E. R., Hua, L.: Discovery of a mandelonitrile hydrolase from *Bradyrhizobium japonicum* USDA110 by rational genome mining. *J Biotechnol* **129**, 645-650 (2007)

Supplementary Data

a)

CATATGGCGACCACCATCAAGGTTGCGGTGACCCAGGCTGAACCGATCTGGCTGGATCTGCAGGCGAGCATCCAGAA
AGCGGTTTCTCTGGTTCACGAAGCTGCGTCTAACGGTGCGAAAATCGTTGCGTTCTCCGAGACCTGGGCACCGGGTT
ACCCGGGCTGGTGTTGGGCGCGTCCGGTTGATCCGGCGCTGAACACCAAATATGCGTACAACTCTCTGACTGCGAAC
TCCCCGGAGATGGAACAGCTGCAGCAGGCGGCTAAAGAGGACAGCATCGCGGTTGTGATCGGCTTCTCTGAACGTAG
CTCTAGCGGCTCCCTGTATATTGGTCAGGCTATCATTTCTCCGCAGGGCGAGGTTGCTCTGCAGCGTCGCAAGCTGA
AACCGACCCACATGGAGCGTACCATTTTCGGCGACGGCTCTGGCCCGGATCTGAACTGTGTGGCGGAGCTGGACTTT
GGCAGCGAACTGGGCTCTATCAAAGTTGGCACCCTGAACTGCTGGGAGCACGCGCAGCCGCTGCTGAAATTCCACGA
AATCCAGCAGGGTGTTGTTATTCACATTGCGATGTGGCCGCCGATCGACCCGTATCCGGGTGTGGAGTTCCCGGGTC
TGTGGTCTATGACCGCGGACGGCTGCCAGAACCTGTCTCAGACCTTTGCGGTTGAATCTGGTGCGTTCGTTCTGCAT
TGTACCGCTGTTTGCAACGAGTCTGGTATCGAAGCGATGGACACCCGTAACGGTATGGTTTTCCGTGAGCCGGGTGG
TGGCCATTCTTGCGTTATCGGTCCGGATGGTCGTCGTCTGACTCAGCCGCTGGCGGATAAACCGTCTGCGGAAGGTA
TCGTTTACGCGGACCTGGACCTGACCCGTGTGGTTACCAACAAATCTTTCCAGGACATCGTTGGTCACTACTCCCGC
CCGGACCTGCTGTGGCTGTCTTACGACAAAGAGAAGAAAGATGCGGCGGTGCATCGTAACTGAAAGCTT

b)

CATATGGCAACAACGATCAAAGTCGCAGTCACACAGGCCGAGCCGATCTGGCTGGATCTTCAGGCGTCCATCCAGAA
AGCGGTATCGCTCGTCCACGAAGCCGCAAGCAATGGCGCAAAGATCGTTGCATTTTCCGAGACATGGGCCCCGGGCT
ACCCTGGTTGGTGTTGGGCCCGACCGGTCGACCCGGCGCTGAACACGAAGTATGCGTACAACTCCCTCACAGCCAAC
TCGCCCGAGATGGAGCAGCTCCAACAAGCTGCCAAGGAAGATTCAATCGCGGTCGTCATCGGCTTCTCGGAGCGCAG
CTCTTCCGGCAGCCTGTACATTGGACAGGCCATCATCTCGCCCCAGGGAGAGGTGGCGTTGCAACGCCGCAAGCTCA
AGCCGACGCACATGGAACGCACAATCTTCGGCGACGGGTCCGGCCCGGACCTCAACTGCGTCGCAGAGCTCGACTTC
GGGTCGGAGCTGGGGTCCATCAAGGTCGGCACGCTCAACTGCTGGGAACACGCTCAGCCGCTCCTCAAGTTCCACGA
GATCCAGCAGGGCGTCGTCATTCATATCGCCATGTGGCCACCCATCGACCCCTACCCGGGCGTCGAATTTCCGGGTC
TGTGGAGCATGACGGCCGACGGGTGCCAGAACCTGTCGCAGACGTTCGCCGTCGAGAGCGGCGCGTTCGTGCTGCAC
TGCACCGCCGTGTGCAACGAGAGCGGCATCGAGGCCATGGACACGCGCAACGGCATGGTGTTCCGCGAGCCCGGCGG
CGGCCACAGCTGCGTCATTGGCCCCGACGGGCGACGCCTTACGCAACCCCTGGCCGACAAGCCGAGCGCCGAGGGTA
TCGTGTATGCCGACCTGGACCTGACCAGGGTGGTGACCAACAAGAGTTTCCAGGATATCGTCGGCCACTACAGCCGC
CCCGACCTCCTCTGGCTTTCGTACGATAAGGAGAAGAAGGATGCTGCGGTACATCGGAACTAAAAGCTT

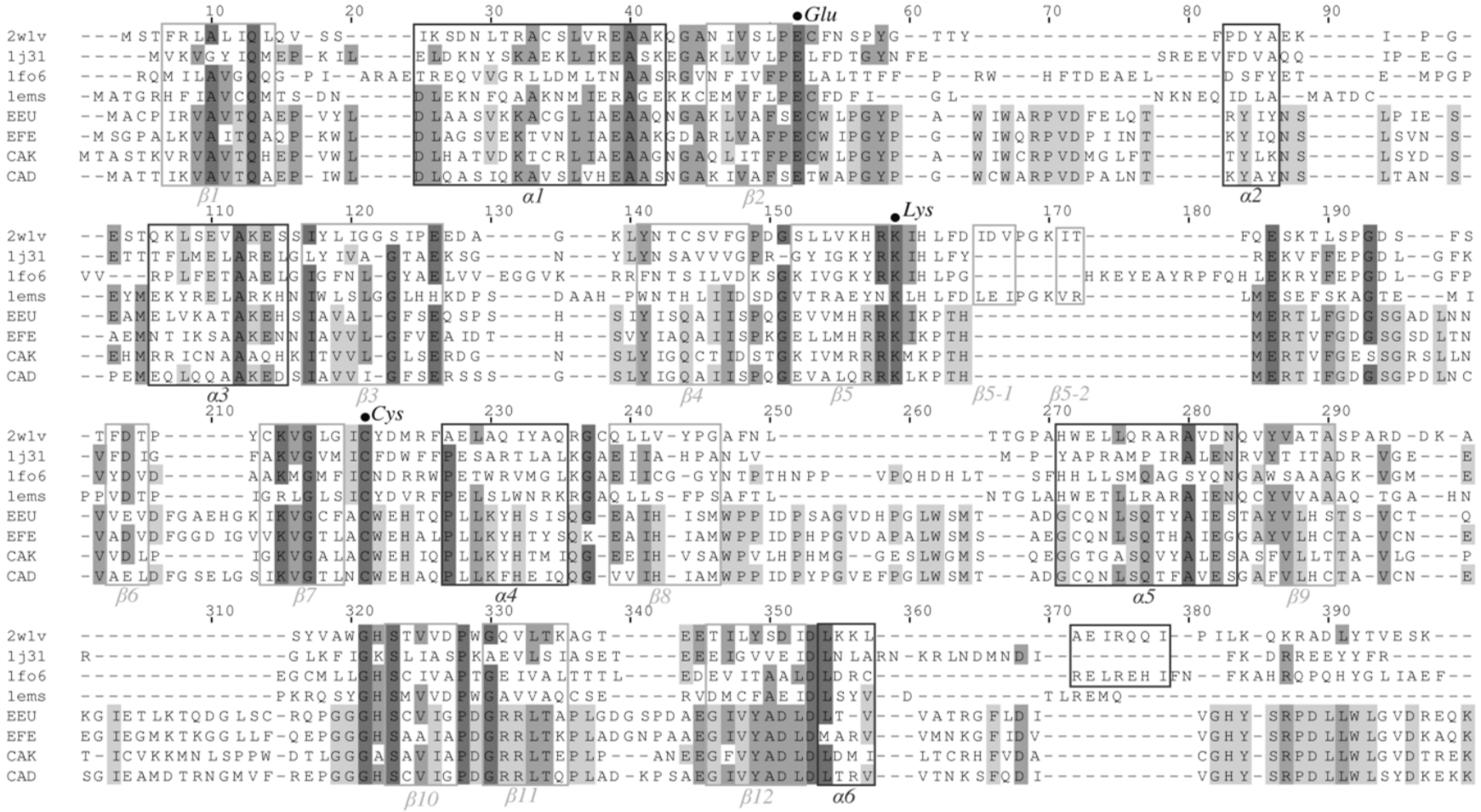
c)

TGAGGCCAGTTAATTAAGAGGTACCATATGGCAACCACCATTAAAGTTGCAGTTACCCAGGCAGAACCGATTTGGCT
GGATCTGCAGGCAAGCATTCAGAAAGCAGTTAGCCTGGTTCATGAAGCAGCAAGCAATGGTGCAAAAATTGTGGCAT
TTAGCGAAACCTGGGCACCGGGTTATCCGGGTTGGTGTTGGGCACGTCCGGTTGATCCGGCACTGAATACCAAATAT
GCCTATAATAGCCTGACCGCAAATTCTCCGGAAATGGAACAGCTGCAGCAGGCAGCAAAAGAAGATAGCATTGCCGT
TGTGATTGGTTTTAGCGAACGTAGCAGCAGCGGTAGCCTGTATATTGGTCAGGCAATTATTTCTCCGCAGGGTGAAG
TTGCACTGCAGCGTCGCAAACTGAAACCGACCCACATGGAACGTACCATTTTTGGTGATGGTAGCGGTCCGGATCTG
AATTGTGTTGCCGAACTGGATTTTGGTAGCGAACTGGGCAGCATTAAAGTTGGCACCCTGAATTGTTGGGAACATGC
ACAGCCGCTGCTGAAATTCCACGAGATCCAACAGGGTGTGGTTATTCATATTGCAATGTGGCCTCCGATTGATCCGT
ATCCGGGTGTTGAATTTCCGGGTCTGTGGAGCATGACCGCAGATGGTTGTCAGAATCTGAGCCAGACCTTTGCAGTT
GAAAGCGGTGCATTTGTTCTGCATTGTACCGCAGTTTGTAATGAAAGCGGCATTGAAGCAATGGATACCCGTAATGG
TATGGTTTTTCGTGAACCGGGTGGTGGTCATAGCTGTGTTATTGGTCCGGATGGTCGTCGTCTGACCCAGCCGCTGG
CCGATAAACCGAGCGCAGAAGGTATTGTTTACGCCGATCTGGATCTGACCCGTGTTGTTACCAATAAATCCTTTCAG
GATATTGTGGGTCATTATTCTCGTCCAGACCTGCTGTGGCTGTCTTATGACAAAGAAAAAAAAGATGCAGCCGTGCA
TCGTAATTAATAAAAGCTTGAGCTCATGGCGCGCCTAGGCCTT

Supplementary figure S1: Nucleotide sequences of *Neurospora crassa* genes: a) gene sequence optimized by Generay, b) non optimized gene sequence, c) gene sequence optimized by GeneArt

```
uniprot:A.niger_CBS_513.88(CAK46742).      ----------------------------MTASTKVRVAVTQHEPVWLDLHATVDKTCRLI
uniprot:A.niger_CBS_513.88(CAK47246).      -----------------------------MLSQVRVAVTQAEPVWLDLEATVKKTCDLI
uniprot:A.oryzae_RIB40(BAE63579).          -------------------------MTTPQPSQVRVAVTQAEPVWLDLKATVDKTCSLI
uniprot:N.crassa_OR74A0(CAD70472).         MPASLKEVAPKQKQTVRINSLPFLSQPQETMATTIKVAVTQAEPIWLDLQASIQKAVSLV
                                                                  : ::***** **:****.*::.*:  *:

uniprot:A.niger_CBS_513.88(CAK46742).      AEAAGNGAQLITFPECWLPGYPAWIWCRPVDMGLFTTYLKNSLSYDSEHMRRICNAAAQH
uniprot:A.niger_CBS_513.88(CAK47246).      AEAAANGAQLVTFPECWIPGYPAWIWARPVDMRLSSIYIQNSLKIDSPQMASIQQCAAEN
uniprot:A.oryzae_RIB40(BAE63579).          AEAASKGAQLVSFPECWIPGYPAWIWTRPVDQELHSRYIQNSLTVSSPEMTQICKSANEN
uniprot:N.crassa_OR74A0(CAD70472).         HEAASNGAKIVAFSETWAPGYPGWCWARPVDPALNTKYAYNSLTANSPEMEQLQQAAKED
                                            ***.:**::::* * * ****.* * ****  * : *  ***. .* .*  : :.* :.

uniprot:A.niger_CBS_513.88(CAK46742).      KITVVLGLSERD-GNSLYIGQCTIDSTGKIVMRRRKMKPTHMERTVFGESSGRSLLNVVD
uniprot:A.niger_CBS_513.88(CAK47246).      KIVVVLGFSENL-HNSLYISQAIIASDGKILTTRKKIKPTHMERTIFGDSFGDCLQSVVD
uniprot:A.oryzae_RIB40(BAE63579).          NVIVVLGFSENI-HNSLYISQAIISNTGSILTTRKKIKATHMERTIFGDAFADCLDSVVE
uniprot:N.crassa_OR74A0(CAD70472).         SIAVVIGFSERSSSGSLYIGQAIISPQGEVALQRRKLKPTHMERTIFGDGSGPDLNCVAE
                                           .: **:*:**.    ****.*. *   *.:   *:*:* ******:**:. .  *  *.:

uniprot:A.niger_CBS_513.88(CAK46742).      LPIG------KVGALACWEHIQPLLKYHTMIQGEEIHVSAWPVLHPHMG--GESLWGMSQ
uniprot:A.niger_CBS_513.88(CAK47246).      TSAG------RVGALSCWEHIQPLLKYHTYAQREQIHVAAWPPLFPH--SEDGSLFSMST
uniprot:A.oryzae_RIB40(BAE63579).          TAVG------RVGALSCWEHIQPLLKYHTCAQREAIHVAAWPPLFEWGGPEDESLFSMSR
uniprot:N.crassa_OR74A0(CAD70472).         LDFGSELGSIKVGTLNCWEHAQPLLKFHEIQQGVVIHIAMWPPIDPYPGVEFPGLWSMTA
                                              *      :**:* **** *****:*   *   **:: ** :         .*:.*:

uniprot:A.niger_CBS_513.88(CAK46742).      EGGTGASQVYALESASFVLLTTAVLGPTCVKKMNLSP--PWDTLGGGASAVIAPDGRRLT
uniprot:A.niger_CBS_513.88(CAK47246).      EGTSSIARTYAIESQSFVLHTTTVIGQSGIDRMATSTGALMSTPGGGCSAIFGPDGRQLS
uniprot:A.oryzae_RIB40(BAE63579).          DGTIALARTYAIESSSFVLHTTAVISQEGVEKMRTATGAIMNMPGGGSSAIFGPDGRLLS
uniprot:N.crassa_OR74A0(CAD70472).         DGCQNLSQTFAVESGAFVLHCTAVCNESGIEAMDTRNGMVFREPGGGHSCVIGPDGRRLT
                                           :*    ::.:*:** :***  *:*     :. *           *** *.::.**** *:

uniprot:A.niger_CBS_513.88(CAK46742).      EPLP--ANEEGFVYADLDLDMILTCRHFVDACGHYSRPDLLWLGVDTREKTQHRPEGQAD
uniprot:A.niger_CBS_513.88(CAK47246).      QPIP--SAEEGIIYADLDFEHIYHSKAFVDVCGHYSRPDLLWLGVEGGVKRHVRDNATTA
uniprot:A.oryzae_RIB40(BAE63579).          KPLL--PTEEGIIYADLEMHDIYKTKAFVDVLGHYSRPDLLWLGVGSCDRRHVKEDAEER
uniprot:N.crassa_OR74A0(CAD70472).         QPLADKPSAEGIVYADLDLTRVVTNKSFQDIVGHYSRPDLLWLSYDKEKKDAAVHRN---
                                           :*:      **::****::  :   : * *  ***********.     :

uniprot:A.niger_CBS_513.88(CAK46742).      NAAYGLDVPSGLVEEEGA
uniprot:A.niger_CBS_513.88(CAK47246).      TPQVEQ-Q------EE--
uniprot:A.oryzae_RIB40(BAE63579).          REDRVE-V------L---
uniprot:N.crassa_OR74A0(CAD70472).         ------------------
```

Supplementary figure S2: Amino acid sequences of expressed nitrilases from *Aspergillus niger* CBS 513.88*, Aspergillus oryzae* RIB40 and *Neurospora crassa* OR74A



Supplementary figure S3: Multiple sequence alignment: mouse nitrilase (2w1v), *Pyrococcus horikoshi* nitrilase (1j31), bacterial amidohydrolase from *Agrobacterium radiobacter* (1fo6), NitFHit from *Caenorhabditis elegans* (lems), nitrilase from *Nectria haematococca* mpVI 77-13-4 (EEU), nitrilase from *Arthroderma benhamiae* CBS 112371 (EFE), nitrilase from *Aspergillus niger* CBS 513.88 (CAK), nitrilase from *Neurospora crassa* OR74A (CAD)

List of publications

Articles related to the presented thesis

Kaplan, O., Bezouška, K., Malandra, A., Veselá, A. B., **Petříčková, A.**, Felsberg, J., Rinágelová, A., Křen, V., Martínková, L.: Genome mining for the discovery of new nitrilases in filamentous fungi. *Biotechnol Lett* **33**, 309-312 (2011)

Petříčková, A., Veselá, A. B., Kaplan, O., Kubáč, D., Uhnáková, B., Malandra, A., Felsberg, J., Rinágelová, A., Weyrauch, P., Křen, V., Bezouška, K., Martínková, L.: Purification and characterization of heterologously expressed nitrilases from filamentous fungi. *Appl Microbiol Biotechnol* **93**, 1553-1561 (2012)

Petříčková, A., Sosedov, O., Baum, S., Stolz, A., Martínková, L.: Influence of point mutations near the active site on catalytic properties of fungal arylacetonitrilases from *Aspergillus niger* and *Neurospora crassa. J Mol Catal B: Enzym* **77**, 74-80 (2012)

Kaplan, O., Veselá, A. B., **Petříčková, A.**, Pasquarelli, F., Pičmanová, M., Rinágelová, A., Bhalla, T. C., Pátek, M., Martínková, L.: A comparative study of nitrilases identified by genome mining. *Mol Biotechnol* **54***,* 996-1003 (2013)

Other research articles

Uhnáková, B., **Petříčková, A.**, Biedermann, D., Homolka, L., Vejvoda, V., Bednář, P., Papoušková, B., Šulc, M., Martínková, L.: Biodegradation of brominated aromatics by cultures and laccase of *Trametes versicolor*. *Chemosphere* **76**, 826-832 (2009)

Uhnáková, B., Ludwig, R., Pěknicová, J., Homolka, L., Lisá, L., Šulc, M., **Petříčková, A.**, Elzeinová, F., Pelantová, H., Monti, D., Křen, V., Haltrich, R., Martínková, L.: Biodegradation of tetrabromobisphenol A by oxidases in basidiomycetous fungi and estrogenic activity of the biotransformation products. *Bioresour Technol* **102**, 9409-9415 (2011)

Veselá, A., **Petříčková, A.**, Weyrauch, P., Martínková, L.: Heterologous expression, purification and characterization of arylacetonitrilases from *Nectria haematococca* and *Arthroderma benhamiae*. *Biocatal Biotransform* **31**, 49-56 (2013)

Oral presentations

Petříčková A.: Expression and characterization of nitrilases from filamentous fungi. Annual seminar of project GACR 305/09/H008 „Preparation, biotransformation and optimalization of compounds with antitumour and antimicrobial effects“, Jáchymov, (10. – 13.6.2010)

Petříčková A.: Structure and biocatalytic properties of nitrilases from filamentous fungi. Annual seminar of project GACR 305/09/H008 „Preparation, biotransformation and optimalization of compounds with antitumour and antimicrobial effects“, Nové Hrady, (9. – 11.6.2011)

Poster presentations

Petříčková, A., Uhnáková, B., Vejvoda, V., Rázga, M., Homolka, L., Martínková, L.: Biodegradace bromovaných aromatických látek. XXI. Biochemický sjezd, České Budějovice (14. – 17.9.2008)

Petříčková, A., Martínková, L., Kaplan, O., Veselá, A. B., Malandra, A., Rinágelová, A., Benada, O., Křen, V., Bezouška, K.: Mining of sequence data for nitrilases in filamentous fungi: expression and characterization of nitrilases in *Aspergillus, Gibberella, Neurospora* and *Penicillium* genera. 5th International Congress on biocatalysis, Hamburg, Germany (29.8.2010 – 2.9.2010)

Petříčková, A., Sosedov, O., Veselá, A. B., Kaplan, O., Stolz, A., Martínková, L.: Influence of point mutations near the active site on catalytic properties of fungal arylacetonitrilases from *Aspergillus niger* and *Neurospora crassa*. Recombinant protein production 6, Vienna, Austria (16. – 19.2.2011)

Petříčková, A., Veselá, A. B., Martínková, L.: Conversion of (*R,S*)-mandelonitrile in water–toluene biphasic system by recombinant nitrilases from *Aspergillus niger* and *Neurospora crassa.* Multistep enzyme-catalyzed processes, Graz, Austria (10. – 13.4.2012)

External fellowship

„Mutations of fungal nitrilases“ (2.9. – 3.12.2010), Institute of Microbiology, University of Stuttgart, Germany. Supported by European Cooperation in Science and Technology (COST); action CM0701 (short-term scientific mission fellowship COST-STSM-CM0701-6753).