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Review

Nitrile metabolism in fungi: A review of its key enzymes nitrilases with focus on their biotechnological impact

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ABSTRACT

Nitriles are abundant in the plant kingdom. The ability to detoxify them is beneficial for microbes living in the plant environment. Nitrilases (NLases; EC 3.5.5.-), which hydrolyze nitriles to carboxylic acids, have been well characterized in bacteria, and classified into various substrate-specificity subtypes (aromatic NLases, aliphatic NLases, arylacetoNLases). NLases also occur in filamentous fungi, mainly in *Ascomycota* (subdivision *Pezizomycotina*), as documented by genome mining. However, the investigation of NLases in fungi has been delayed compared to bacteria. Only a few NLases (aromatic NLases) were purified from native fungal strains (mainly *Fusarium*), which were grown under suitable induction conditions. Over a few past years, the spectrum of known fungal NLases was broadened by expressing fungal NLase genes in *Escherichia coli*. Thus functional NLases were reported for the first time in fungi of genera *Auricularia*, *Macrophomina*, *Nectria*, *Neurospora*, *Pichia*, *Talaromyces*, *Trichoderma* and *Trichophyton*. Two major substrate-specificity subtypes were identified in them, i.e. aromatic NLases and arylacetoNLases, apart from a few NLases with broad substrate specificities. The biotechnological impact of fungal arylacetoNLases was explored with a focus on the enantioselective hydrolysis of (*R*,*S*)-mandelonitrile, the selective hydrolysis of one cyano group in dinitriles and the hydrolysis of nitrile precursors of the taxol sidechain. Despite recent advances, the wealth of fungal NLases whose sequences have been deposited in databases has not yet been fully exploited. Overproduction in *E. coli* has the potential to bring these NLases to life. This will enable to estimate the natural roles of NLases in fungi and will also provide new catalysts for biotechnological uses.

1. Introduction

Nitriles are abundant in nature, mainly occurring as cyanogenic glycosides (defense compounds) or β-cyano alanine (HCN assimilation intermediate) in plants. The enzymes of nitrile metabolism are widespread but not ubiquitous in nature and consist of nitrile-forming and nitrile-converting enzymes (Fig. 1). The former are aldoxime dehydratases (Oxds; EC 4.99.1.5–7) in bacteria and fungi, and cytochrome P450 (CYP) enzymes in plants. Both of them catalyze the dehydration of nitrile precursors, aldoximes, which, in turn, are derived from amino acids. The knowledge of Oxds in fungi is limited, as

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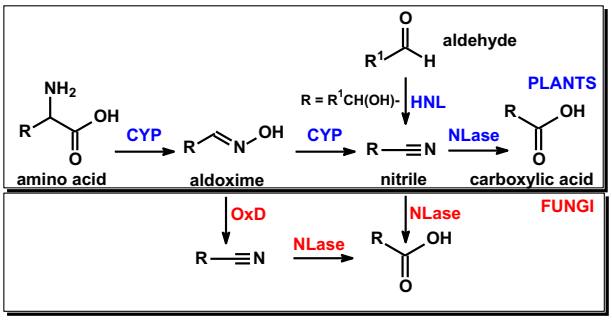

Fig. 1 – Biosynthesis and bioconversion of nitriles in plants and fungi. CYP = cytochrome P450 enzymes; OxD = aldoxime dehydratase; NLase = nitrilase; HNL = hydroxynitrile lyase.

only two fungal Oxds were characterized in some detail. One of them was isolated from the mycelium of *Sclerotinia sclerotiorum* and the other, from *Fusarium graminearum*, was overproduced in *E. coli*. The former transformed a few aldoximes such as indolyl-3-acetaldoxime, 4-hydroxyphenylacetaldoxime and analogues (Pedras *et al.*, 2010), while the latter exhibited a broad substrate specificity for aliphatic and arylaliphatic aldoximes (Kato and Asano 2005).

Some specific nitriles (cyanohydrins in plants, toyocamycin in *Streptomyces*) are synthesized by other nitrile-forming enzymes. The synthesis of cyanohydrins from aldehydes and HCN is catalyzed by hydroxynitrile lyases (HNLs; 4.1.2.-), which have also found a number of applications in the synthesis of chiral nitriles (for reviews see Bracco *et al.* 2016 and Bhalla *et al.* 2018). The nucleosidic antibiotic toyocamycin is produced via its nitrile precursor PreQ(0) (7-cyano-7-deazaguanin; Tao *et al.*, 2015), while 7-cyano-7-deazaguanine synthetase (EC 6.3.4.20) participates in the ATP dependent synthesis of the nitrile precursor (McCarty *et al.*, 2009; Liu *et al.*, 2018).

The key enzymes of the nitrile catabolism in fungi and plants are NLases (EC 3.5.5.-). The probable roles of NLases in plants consist of e.g. cyanide detoxification, catabolism of natural nitriles, supply of nitrogen for growth, and, under specific conditions, production of the growth hormone indole-3-acetic acid (for a review see Piotrowski, 2008). The natural functions of NLases in microbes are less understood but it is justified to hypothesize on their key roles in nitrile detoxification and nitrogen recycling, which may facilitate the organisms' participation in dead plant decay or plant pathogenesis. In addition to NLases, bacteria produce an alternative enzymatic system enabling the hydrolysis of nitriles in two steps: 1. transformation of nitrile to amide by nitrile hydratase, EC 4.2.1.84, and 2. transformation of amide to carboxylic acid by amidase, EC 3.5.1.4 (for reviews see Kobayashi and Shimizu 1999; Martínková and Křen 2002; Prasad and Bhalla 2010).

NLases are classified into branch 1 of the "NLase-superfamily" proteins, typified by their E-K-E-C catalytic tetrade (Thuku *et al.* 2009). The NLase superfamily consists of 13 branches, whose members are largely known to act on non-peptide C–N bonds, with different substrate specificities. The NLase activity was only found in branch 1 (Brenner 2002).

In the hypothetical reaction mechanism of NLases, the catalytic cystein interacts with the carbon atom of the C≡N moiety, and a tetrahedral intermediate is thus formed, which is then cleaved into carboxylic acid or amide (Fig. 2). The former is largely the major product of the reaction, while the latter is formed from specific substrates such as those bearing electrophilic substituents at α-position. However, the tendency to form amide also depends on the origin of the enzyme (Sosedov and Stolz 2014).

Microbial NLases have been usually classified into a few subtypes (aromatic NLases, arylacetoNLases and aliphatic NLases) designated according to their preferential substrates (aromatic nitriles, arylacetonitriles, aliphatic nitriles; Kobayashi and Shimizu 1994; O'Reilly and Turner, 2003; Thuku *et al.*, 2009). The latter study also defined the bromoxynil-specific NLases (Thuku *et al.*, 2009). Plant NLases are comprised of subtypes NIT1-3 with preference for some arylaliphatic and aliphatic nitriles and NIT4 with preference for β-cyanoalanine (Vorwerk *et al.*, 2001; Piotrowski *et al.*, 2001).

The interest in NLases has been prompted by their ability to hydrolyze nitriles under ambient conditions and at mild pH values, to discriminate between enantio- and regioisomers, and to selectively hydrolyze cyano groups in presence of other functional groups. These biotransformations were mainly examined with the bacterial NLases (for reviews see Martínková and Křen, 2010; Martínková *et al.*, 2017) and, partly, the plant NLases (Osswald *et al.*, 2002), while fungal NLases have been largely neglected for years.

The first fungal NLase (aromatic NLase) was purified from *Fusarium solani* by Harper (1977) but its amino acid (aa) sequence was never determined. Later, using 2-cyanopyridine and valeronitrile as powerful inducers enabled to isolate two aromatic NLases from this species and to partially determine their sequences (for a review see Martínková *et al.*, 2009). In addition, a NLase with a broad substrate specificity was induced in *Fusarium proliferatum* by ε-caprolactam (Yusuf *et al.*, 2013a, b). However, isolating NLases from fungi has its limits, as the enzyme production may be low, inducer difficult to find or the cultivation of the fungus time consuming. Therefore, heterologous expression has been used in recent years to obtain new fungal NLases. These studies not only resulted in the characterization of further aromatic NLases, but also enabled it to prepare a number of arylacetoNLases, which until that time had only been found in bacteria.

The properties and applications of NLases have been reviewed from many viewpoints. For instance, some

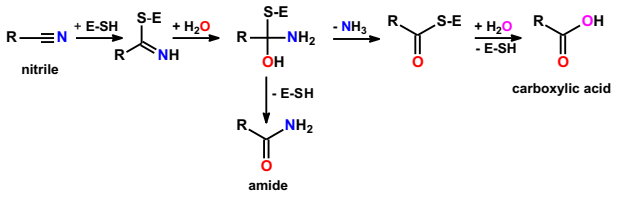

Fig. 2 – Hypothetical reaction mechanism of nitrilase (according to O'Reilly and Turner, 2003). E = enzyme.

comprehensive reviews summarized the knowledge of NLases (Gong *et al.*, 2012b), the nitrile-converting enzymes (Ramteke *et al.*, 2013) or the aldoxime-nitrile pathway (Bhalla *et al.*, 2018). Other reviews focused on specific aspects such as the metagenome and database mining for NLases (Gong *et al.*, 2013), integration of nitrile-hydrolyzing enzymes in cascade reactions (Martínková *et al.*, 2014), biological degradation of cyanide (Luque-Almagro *et al.*, 2016), enantioselective biotransformations of nitriles and amides (Wang, 2015) or NLase applications with industrial impact (Martínková *et al.*, 2017). The focus of these studies was on bacterial enzymes, while the properties or applications of fungal NLases were only marginally discussed. The only review which focused exclusively on fungal NLases was published ca. 9 y ago (Martínková *et al.*, 2009). The heterologous production of these enzymes was in its early stage at that time. Therefore, the aim of this work is to summarize the advances in this research area, i.e. the production methods, the biochemical characterization and the biotechnological uses of the enzymes. The phylogenetic distribution of their subtypes will also be discussed. Cyanide hydratases, which form an evolutionarily distant type of enzymes, and cyanide dihydratases (nitrilase homologues) have been recently reviewed elsewhere (Martínková *et al.*, 2015, Park *et al.*, 2017) and will not be included in this work.

2. Heterologous expression of fungal nitrilase genes and biochemical characterization of the enzymes

Fungal NLases are probably not glycosylated or at least do not require glycosylation for their activities. Therefore, *E. coli* can be used as a suitable host for the expression of fungal NLase genes. Thus the expression systems used for bacterial and fungal NLases were similar. Low concentrations of the inducer IPTG (0.5 mM) and relatively low temperatures (e.g., 25 °C) had to be maintained to avoid excessive translation rates, and, hence, to minimize protein misfolding (Petříčková *et al.*, 2012b). An optimized protocol even used IPTG concentrations as low as 0.02 mM. Both *E. coli* BL21 and *E. coli* Origami B (DE23) were acceptable as the host strains but the latter provided higher activity yields. The genes were cloned into expression vectors of the pET type. To achieve high cell densities, 2 YT and particularly EnPresso® media were suitable (Veselá *et al.*, 2016).

The effect of the co-expression of bacterial chaperones on the production of fungal NLases was examined, and the GroEL/ES chaperone was found to have positive effects on the production of two aromatic NLases. The total activities of these NLases increased almost three to seven times and the specific activities of the whole cells more than four times. At the same time, the percentage of activity in the soluble fractions increased ca. 1.2 to 1.7 times. The effect of this chaperone was enzyme-specific (only efficient in aromatic NLases). Other chaperones such as Hsp70 or trigger factor failed to exhibit similar effects (Petříčková *et al.*, 2012b).

Over 10 of the recombinantly produced fungal NLases were largely purified by metal affinity chromatography (Petříčková *et al.*, 2012b; Gong *et al.*, 2012a; Veselá et al., 2013, 2016; Yusuf *et al.*, 2015). The typical size of their subunits was similar to those in bacterial NLases (29.8–45.8 kDa; Thuku *et al.*, 2009), e.g. 36–37 kDa in NLases from *Trichophyton benhamiae* (formerly *Arthroderma benhamiae*) and *Nectria haematococca* (Veselá *et al.* 2013). The native molecular weights in these NLases were determined to be 336–360 kDa (Veselá *et al.* 2013), suggesting a multimeric structure similar to bacterial NLases with 130–650 kDa (Thuku *et al.*, 2009). All the enzymes examined were moderately thermostable and exhibited pH profiles with maximum activities and stabilities in the neutral or slightly alkaline pH range similar to the majority of bacterial NLases. However, the NLase from *G. moniliformis* was more stable at alkaline pH (up to 11; Petříčková *et al.*, 2012b) than the majority of other NLases (Ramteke *et al.*, 2013), which was in accordance with the high activity of the NLase from *Gibberella intermedia* at pH 10 (Gong *et al.*, 2012a). Similar pH profiles were obtained for the NLase from *T. benhamiae*. The sensitivity of the fungal NLases to Ag^{+} (Gong *et al.*, 2012a; Yusuf *et al.*, 2015) was similar to their bacterial counterparts, reflecting the conserved reaction centre, specifically the key role of the active cystein residue in their reaction mechanism.

3. Substrate specificity subtypes

The spectrum of aromatic NLases, which were previously obtained directly from fungi *Aspergillus niger*, *F. solani*, *Fusarium oxysporum*, *Penicillium multicolor* (for a review see Martínková *et al.*, 2009) and *Exophiala oligosperma* (Rustler *et al.*, 2008), was extended with heterologously produced NLases from *Aspergillus kawachii* (Veselá *et al.*, 2016), *Penicillium rubens* (formerly *Penicillium chrysogenum*) (Kaplan *et al.*, 2013), *Talaromyces marneffei* (formerly *Penicillium marneffei*), *Gibberella moniliformis* (Petříčková *et al.*, 2012b; Kaplan *et al.*, 2013) and *Gibberella intermedia* (*Gibberella fujikuroi* var. *intermedia*) (Gong *et al.*, 2012a). The designation of this subtype as aromatic NLases is based on the finding that aromatic or heteroaromatic nitriles (often 4-cyanopyridine) are their preferential substrates but their ability to hydrolyze straight-chain aliphatic nitriles or dinitriles was also demonstrated (Goldlust and Bohak, 1989; Gong *et al.*, 2012a; Kaplan *et al.*, 2013; Veselá *et al.*, 2016). The turnover number (TON) of the aromatic NLase from *G. moniliformis* for its best substrate benzonitrile was ca. 5.8 s^{-1}, as calculated from kinetic data determined by Petříčková *et al.* (2012b). Thus this value was significantly lower than in NLases from *Rhodococcus rhodochrous* (ca. 129 s^{-1}; Yeom *et al.*, 2010) or even *Aeribacillus pallidus* (formerly *Bacillus pallidus*; 14 444 s^{-1}; Almatawah *et al.*, 1999).

The first two fungal arylacetoNLases were obtained by expressing the genes from *A. niger* and *Neurospora crassa* in *E. coli* (Kaplan *et al.*, 2011; Petříčková *et al.*, 2012b). Aromatic and heteroaromatic nitriles were poor substrates of these enzymes, while the preferential ones were phenylacetonitrile and (*R*,*S*)-mandelonitrile. Similar enzymes were later found to be encoded in *T. benhamiae*, *A. kawachii*, *Aspergillus oryzae*, *Auricularia subglabra* (formerly *Auricularia delicata*), *N. haematococca* and *Macrophomina phaseolina* (Kaplan *et al.*, 2013; Veselá et al., 2013, 2016). The TONs of fungal arylacetoNLases, in which kinetic data were determined (NLases from *A. niger*,

N. crassa, *N. haematococca* and *T. benhamiae*; Petříčková *et al.*, 2012b; Veselá *et al.*, 2013), were calculated to be 6.5–68.5 s^{-1} for phenylacetonitrile and 6.1–19.3 s^{-1} for (*R*,*S*)-mandelonitrile. Those of bacterial NLases covered a broad range of 0.072–175 s^{-1} for mandelonitrile and 1.1–18.85 s^{-1} for phenylacetonitrile (brenda-enzymes.org). Thus the TONs of fungal NLases were largely in this range except for the TON of *N. haematococca* NLase for phenylacetonitrile, which was the highest (68.5 s^{-1}).

A different NLase with a broad substrate specificity (for aromatic and aliphatic nitriles but also mandelonitrile) was obtained by expressing a gene from *Fusarium proliferatum*. This protein was evolutionarily distant from the aforementioned fungal NLases. Its molecular mass (theoretically 33 kDa) was slightly lower than in the majority of other NLases with typical masses of 35–45 kDa (Yusuf *et al.*, 2015).

Different sequence patterns could be recognized in the proximity of the catalytic cystein in different substrate-specificity subtypes (Fig. 3). For instance, all proteins with a short insert of six aa residues upstream of the catalytic cystein exhibited arylacetoNLase activities. However, there were also other arylacetoNLases lacking this pattern. The broad substrate specificity NLases from *Meyerozyma* contained an insert comprised of five aa residues. Notably, the *F. proliferatum* lacked the typical NLase motif CWEH, containing a CYDV motif instead.

4. Phylogenetic distribution

The aforementioned characterized NLases largely originated from division *Ascomycota* (subdivision *Pezizomycotina*). Similar putative NLases were also predicted in many other members of *Pezizomycotina*. These fungi belonged to the classes *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes* and *Sordariomycetes*. The searches also indicated a rare occurrence of similar NLases in subdivision *Saccharomycotina*. Various types of NLases were found in almost each of the classes, and very often also in the same genus (Table 1).

Close homologues of the NLase from *F. proliferatum* occur in other species of *Fusarium* but also in other genera of subdivision *Pezizomycotina* (*Trichoderma*, *Metarhizium*, *Colletotrichum*, *Penicillium* etc.) and are largely designated carbon-nitrogen hydrolases in databases. This group of NLase family proteins exhibit significant levels of identity to the carbon-nitrogen hydrolase from *Saccharomyces cerevisiae* (ca. 50% in NLase from *F. proliferatum*; Yusuf *et al.*, 2015). The putative hydrolase from *S. cerevisiae* was crystallized (Kumaran *et al.*, 2003) which enabled to use it as the template for modeling and substrate docking studies of the NLase from *F. proliferatum* (Yusuf *et al.*, 2015).

Basidiomycota did not contain any NLases similar to the aforementioned subtypes except for the aforementioned NLase from *Auricularia*. It is possible that the gene was acquired by this fungus through horizontal transfer. The putative NLase superfamily proteins, which are found in *Basidiomycota* (unpublished results), are distant in evolution from the NLases discussed here and their functions or activities are still unclear.

5. Biotechnology potential

In organic synthesis, NLases have been often found to be suitable alternatives of chemical catalysts, operating at mild temperatures and near neutral pH, and, in many cases, in a stereo- or a regioselective mode. However, the industrial applications of NLases are still few, comprising e.g. of the production of pyrazine-2-carboxylic acid, picolinic acid (Liese *et al.*, 2000), nicotinic acid (Gong *et al.*, 2012b) or mandelic acid and its derivatives (Brady *et al.*, 2004; Gong *et al.*, 2012b). Although the first-generation catalysts have been already applied for the industrial synthesis of (*R*)-mandelic acid (synthetic intermediate, chiral resolving agent) from (*R*,*S*)-mandelonitrile, new and improved catalysts of this reaction are being constantly sought. Recently, whole cells of *E. coli* producing fungal arylacetoNLases were found to be suitable candidates for such catalysts. The cells hydrolyzed up to 500 mM mandelonitrile (Veselá *et al.*, 2015), while the

ArylacetoNLase	*Auricularia delicata*	gb|EJD42068.1	FGGEIGTVKVGALACWEHTQPLLK
	Macrophomina phaseolina	gb|EKG14506.1	FGGDVGVVKVGALACWEHTQPLLK
	Trichophyton benhamiae	gb|AFF60190.1	FGGDIGVVKVGTLACWEHALPLLK
	Neurospora crassa	emb|CAD70472.1	FGSELGSIKVGTLNCWEHAQPLLK
	Nectria haematococca	gb|AFF60191.1	FGAEHGKIKVGCFACWEHTQPLLK
	Aspergillus niger	gb|AEH52057.1	IG------KVGALACWEHIQPLLK
		emb|CAK47246.1	AG------RVGALSCWEHIQPLLK
	Aspergillus kawachii	dbj|GAA83217.1	AG------RVGALSCWEHIQPLLK
	Aspergillus oryzae	dbj|BAE63579.1	VG------RVGALSCWEHIQPLLK
Aromatic NLase	*Aspergillus kawachii*	dbj|GAA90167.1	FG------NIGGLNCWEHTQTLLR
	Gibberella moniliformis	gb|ABF83489.1	FG------RVAGLNCWEHTQTLLR
	Penicillium rubens	gb|KZN90726.1	FG------RIGGLNCWEHTQPLLR
	Talaromyces marneffei	gb|AEH52060.1	FG------KVGGLNCWEHLQPLLR
Broad-substrate -specificity NLases	*Meyerozyma guilliermondi*	gb|AFF60192.1	FK-EAGPVEVGCLSCWEHMQPLLY
	Trichoderma virens	gb|EHK18468.1	FG------RVGSLSCWEHIQPLLK
	Fusarium proliferatum	gb|AGW81831.1|	YG------KIAVAICYDVRFPELA

Fig. 3 – Multiple alignment of sequences surrounding catalytic region in fungal nitrilases; NLase = nitrilase. Identical residues are highlighted in red. Strongly similar residues and weakly similar residues are in blue and green, respectively. The catalytic cystein is underlined.

Table 1 – Distribution of aromatic nitrilase, arylacetonitrilase and broad-substrate-specificity nitrilase homologues in filamentous fungi.

Division	Subdivision	Class	Genera[a] containing		
			Aromatic NLases	ArylacetoNLases	Broad-substrate-specificity NLases
Ascomycota	*Pezizomycotina*	*Dothideomycetes*	*Cercospora*, *Clohesyomyces*, *Neofusicoccum*, *Pyrenochaeta*, *Verruconis*	*Baudoinia*, *Cenococcum*, *Cercospora*, *Clohesyomyces*, *Diplodia*, *Glonium*, ***Macrophomina***, *Neofusicoccum*, *Paraphaeosphaeria*, *Pyrenochaeta*	
		Eurotiomycetes	***Aspergillus***, *Capronia*, *Cladophialophora*, *Exophiala*, *Penicilliopsis*, ***Penicillium***, *Phialophora*, ***Talaromyces***	***Aspergillus***, *Capronia*, *Cladophialophora*, *Emmonsia*, *Exophiala*, *Microsporum*, *Nannizzia*, *Paracoccidioides*, *Penicilliopsis*, *Penicillium*, *Phialophora*, *Talaromyces*, ***Trichophyton***	
		Leotiomycetes	*Coleophoma*, *Oidiodendron*, *Pezoloma*, *Phialocephala*	*Botrytis*, *Coleophoma*, *Hyaloscypha*, *Oidiodendron*, *Pezoloma*, *Phialocephala*, *Pseudogymnoascus*, *Rutstroemia*, *Sclerotinia*	
		Sordariomycetes	*Colletotrichum*, Daldinia, *Diaporthe*, ***Fusarium***, ***Gibberella***,Hypoxylon, Nectria, *Neonectria*, *Pestalotiopsis*, *Phaeoacremonium*, *Rosellinia*, *Tolypocladium*, *Verticillium*	*Colletotrichum*, *Daldinia*, *Diaporthe*, *Fusarium*, *Hypoxylon*, *Nectria*, ***Neonectria***, ***Neurospora***, *Pestalotiopsis*	***Trichoderma***
	Saccharomycotina	*Saccharomycetes*			***Meyerozyma***, *Wickerhamomyces*
Basidiomycota		*Agaricomycetes*		***Auricularia***	

a The genera in which NLases were experimentally confirmed (Rustler *et al.*, 2008; Kaplan *et al.*, 2013; Veselá *et al.*, 2016) are in bold. Other genera shown contain homologues with at least 50% identity to experimentally confirmed NLases.

bacterial NLase was already inhibited by 300 mM of this substrate and toluene (10%, v/v) had to be added to alleviate this inhibition (Zhang *et al.*, 2011). The tolerance of fungal NLase towards high product concentrations was also significant, the reaction rate being almost constant at up to ca. 500 mM of (*R*)-mandelic acid.

All fungal NLases examined were selective for (*R*)-mandelonitrile, but their selectivities were of different degrees. The NLases from e.g. *A. niger*, *N. crassa* or *N. haematococca* exhibited high selectivity for (*R*)-mandelonitrile. The hydrolysis of (*R,S*)-mandelonitrile into (*R*)-mandelic acid must be performed under conditions of rapid racemization of the substrate (pH $\geq$ 8). This ensures that the "correct" enantiomer (*R*-nitrile) is permanently available for the catalyst and a high enantiopurity of the product is maintained (Fig. 4). The ee values ($\leq$95.6%) obtained with fungal NLases acting on high substrate concentrations were slightly lower than with bacterial NLases (up to ca. 99%) but ee can be increased by optimizing the substrate feed or by recrystallization (Zhang *et al.*, 2010). In contrast, the catalyst productivity of the fed-batch process (ca. 40 g g^{-1}) was largely higher than with non-recycled bacterial NLases. This allowed low catalyst loads of ca. 2 g of dry cell weight L^{-1} to be used (Veselá *et al.*, 2015).

In contrast to the aforementioned NLases, the NLase from *T. benhamiae* was only moderately selective (Veselá *et al.*, 2015; Fig. 4). Its ability to also hydrolyze (*S*)-mandelonitrile can be used for the production of (*S*)-mandelic acid from this substrate under stereoretentive conditions, i.e. a low pH hindering substrate racemization. (*S*)-Mandelonitrile can be obtained in the *S*-oxynitrilase-catalyzed reaction of benzaldehyde and HCN (Chmura *et al.*, 2013).

There is also a great demand for NLases catalyzing the hydrolysis of a single cyano group in dinitriles, as the resulting cyano acids or cyano amides are useful synthons (Bayer *et al.*, 2011; Vergne-Vaxelaire *et al.*, 2013). Whole cells producing fungal arylacetoNLases transformed cyanophenylacetonitriles and phenylenediacetonitriles into the corresponding cyano acids (Fig. 5) at a preparative scale. A member of the aromatic NLase subtype (NLase from *A. kawachii*) exhibited lower activities for these substrates. In contrast, its activity for fumaronitrile was higher than in arylacetoNLases. Different from arylacetoNLases, the aromatic NLase transformed this substrate not into cyano acid, but cyano amide (Fig. 5).

E. coli whole cells producing fungal arylacetoNLases were also used in the chemo-enzymatic synthesis of taxol derivatives with modified side chains (Wilding *et al.*, 2015). Taxol (Paclitaxel) is a well-known anticancer drug, and its derivatives may be useful for studies of its structure–activity relationships. All six NLases examined were active for the *trans*-isomer of the cyanodihydrooxazole precursor (*trans*-1) which they transformed into a mixture of acid and amide. The ratio of both products (7:3 through 9:1) depended on the enzyme. None of these enzymes transformed the corresponding *cis*-isomer (*cis*-1). Thus regioselectivity of the fungal NLases for this type of nitriles was demonstrated (Fig. 6).

Protein engineering was used in several bacterial NLases to improve their catalytic properties (for a review see Martínková *et al.* 2017). The replacements of amino acids in the catalytic region, in particular, were demonstrated to have significant effects on the enantioselectivities, specific activities and amide formation in these enzymes (Kiziak and Stolz, 2009; Sosedov *et al.*, 2010). Although fungal NLases only share moderate similarities with bacterial NLases (with identities largely not exceeding 40%), the region in the proximity of the catalytic centre is more conserved (Kaplan *et al.*, 2013). Therefore, it may be predicted that similar amino acid replacements will change the catalytic properties in bacterial and fungal NLases in a similar way. This was experimentally confirmed by modifying the sequence in the proximity of the catalytic cystein in the arylacetoNLases from *A. niger* and *N. crassa*. In fact, the W168A mutant of the NLase from *N. crassa* gave a much higher amide:acid ratio (85:15) than the wild-type enzyme (40:60) (Petříčková *et al.*, 2012a), which was in accordance with the amide formation by analogous variants of the *Psedomonas fluorescens* NLase (Kiziak and Stolz, 2009). The amide-forming mutants may be useful as alternatives for nitrile hydratases, which

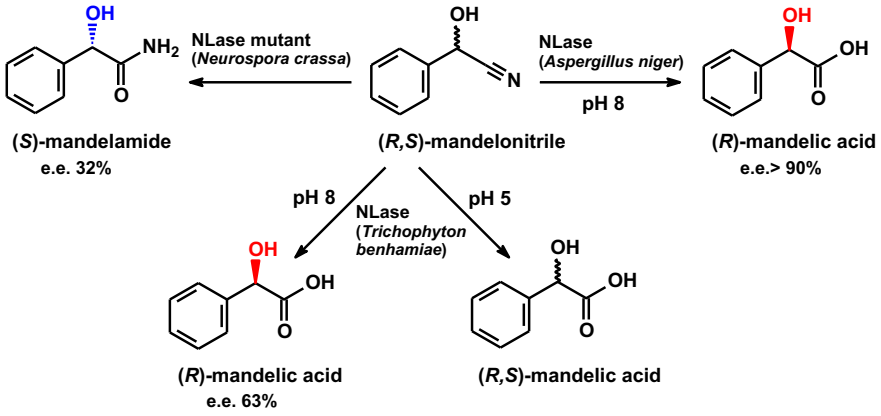

Fig. 4 – Transformations of (*R,S*)-mandelonitrile into different products by various fungal nitrilases (examples); NLase = nitrilase.

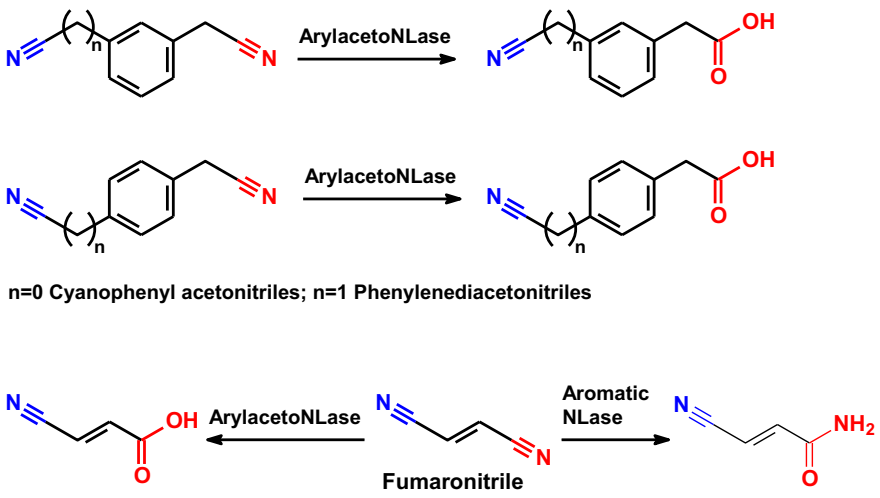

Fig. 5 – Transformations of dinitriles into cyano acids or cyano amide by fungal nitrilases; NLase = nitrilase.

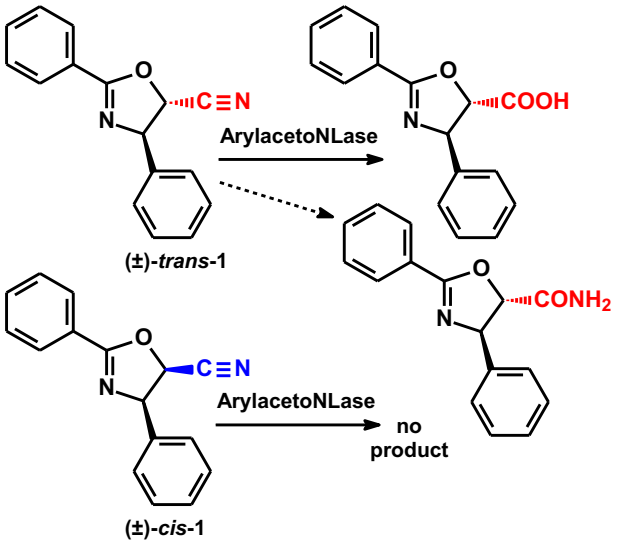

Fig. 6 – Transformation of taxol precursor, (±)-*trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile ((±)-*trans*-1), by fungal nitrilases; (±)-*cis*-1 was not accepted as a substrate. NLase = nitrilase. Only one of the enantiomers is depicted for each compound.

form amides from nitriles, but are largely less stable and less enantioselective than NLases.

6. Conclusions

Fungal NLases can be classified into two main subtypes – aromatic NLases and arylacetoNLases. Although aromatic NLases generally hydrolyze not only aromatic but also aliphatic nitriles, the designation is justified, as their significant activities for aromatic nitriles such as benzonitrile make them different from arylacetoNLases. Another criterion to distinguish between both types is the arylacetoNLase activity for α-substituted arylacetonitriles such as mandelonitrile. Hypothetical members of both subtypes were found to be abundant in specific taxa of division *Ascomycota* and ca. 15 of them were overproduced and characterized. In contrast, the arylacetoNLase from *A. subglabra* was the only characterized NLase from *Basidiomycota*.

Databases contain a wealth of sequences coding for putative NLases with unknown properties. It seems worth unravelling the substrate specifities and other biochemical properties of these proteins, as this may provide new catalysts but also help to elucidate the roles of these enzymes in the metabolism of nitriles in nature. Knowledge of the structure–activity relationships enables us to predict the properties of some of the putative enzymes, close homologues of which were characterized. However, other of these putative enzymes exhibit low similarities to characterized NLases. This is the case for the majority of NLases in *Basidiomycota*. These proteins must be overproduced to determine their functions. The methods for the heterologous production of fungal NLases were established in the aforementioned works and will be useful in further studies of the related enzymes, although optimum conditions will have to be fine-tuned for each new member of this enzyme group. Fungal NLases enlarged the portfolio of NLases with possible applications for synthetic uses but their industrial potential for e.g. the cyanohydrin or dinitrile hydrolysis will require further evaluation, including the preparation of immobilized cells or enzymes and optimization of the process conditions on lab and pilot scale.

Conflict of interest

None.

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REFERENCES

Almatawah, Q.A., Cramp, R., Cowan, D.A., 1999. Characterization of an inducible nitrilase from a thermophilic bacillus. Extremophiles 3, 283–291.

Bayer, S., Birkenmeyer, C., Ballschmiter, M., 2011. A nitrilase from a metagenomic library acts regioselectively on aliphatic dinitriles. Appl. Microbiol. Biotechnol. 89, 91–98.

Bhalla, T.C., Kumar, V., Kumar, V., Thakur, N., Savitri, 2018. Nitrile metabolizing enzymes in biocatalysis and biotransformation. Appl. Biochem. Biotechnol. 185, 925–946.

Bracco, P., Busch, H., von Langermann, J., Hanefeld, U., 2016. Enantioselective synthesis of cyanohydrins catalysed by hydroxynitrile lyases - a review. Org. Biomol. Chem. 14, 6375–6389.

Brady, D., Beeton, A., Zeevaart, J., Kgaje, C., van Rantwijk, F., Sheldon, R.A., 2004. Characterisation of nitrilase and nitrile hydratase biocatalytic systems. Appl. Microbiol. Biotechnol. 64, 76–85.

Brenner, C., 2002. Catalysis in the nitrilase superfamily. Curr. Opin. Struct. Biol. 12, 775–782.

Chmura, A., Rustler, S., Paravidino, M., van Rantwijk, F., Stolz, A., Sheldon, R.A., 2013. The combi-CLEA approach: enzymatic cascade synthesis of enantiomerically pure (*S*)-mandelic acid. Tetrahedron-Asymmetry 24, 1225–1232.

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

Gong, J.S., Li, H., Zhu, X.Y., Lu, Z.M., Wu, Y., Shi, J.S., Xu, Z.H., 2012a. Fungal His-tagged nitrilase from *Gibberella intermedia*: gene cloning, heterologous expression and biochemical properties. PLoS One 7e50622.

Gong, J.S., Lu, Z.M., Li, H., Shi, J.S., Zhou, Z.-M., Xu, Z.H., 2012b. Nitrilases in nitrile biocatalysis: recent progress and forthcoming research. Microb. Cell Fact. 11, e142.

Gong, J.S., Lu, Z.M., Li, H., Zhou, Z.-M., Shi, J.S., Xu, Z.H., 2013. Metagenomic technology and genome mining: emerging areas for exploring novel nitrilases. Appl. Microbiol. Biotechnol. 97, 6603–6611.

Harper, D.B., 1977. Fungal degradation of aromatic nitriles. Enzymology of C-N cleavage by Fusarium solani. Biochem. J. 167, 685–692.

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

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

Kato, Y., Asano, Y., 2005. Purification and characterization of aldoxime dehydratase of the head blight fungus, *Fusarium graminearum*. Biosci. Biotechnol. Biochem. 69, 2254–2257.

Kiziak, C., Stolz, A., 2009. 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.

Kobayashi, M., Shimizu, S., 1994. Versatile nitrilases: Nitrile-hydrolyzing enzymes. FEMS Microbiol. Lett. 120, 217–223.

Kobayashi, M., Shimizu, S., 1999. Cobalt proteins. Eur. J. Biochem. 261, 1–9.

Kumaran, D., Eswaramoorthy, S., Gerchman, S.E., Kycia, H., Studier, F.W., Swaminathan, S., 2003. Crystal structure of a putative CN hydrolase from yeast. Proteins 52, 283–291.

Liese, A., Seelbach, K., Wandrey, C. (Eds), 2000. Industrial biotransformations. Wiley-VCH Verlag, Weinheim.

Liu, Y., Gong, R., Liu, X.Q., Zhang, P.C., Zhang, Q., Cai, Y.S., Deng, Z.X., Winkler, M., Wu, J.G., Chen, W.Q., 2018. Discovery and characterization of the tubercidin biosynthetic pathway from *Streptomyces tubercidicus* NBRC 13090. Microb. Cell Fact. 17, e131.

Luque-Almagro, V.M., Moreno-Vivián, C., Roldán, M.D., 2016. Biodegradation of cyanide wastes from mining and jewellery industries. Curr. Opin. Biotechnol. 38, 9–13.

Martínková, L., Křen, V., 2002. Nitrile- and amide-converting microbial enzymes: Stereo-, regio- and chemoselectivity. Biocat. Biotrans. 20, 73–93.

Martínková, L., Křen, V., 2010. Biotransformations with nitrilases. Curr. Opin. Chem. Biol. 14, 130–137.

Martínková, L., Rucká, L., Nešvera, J., Pátek, M., 2017. Recent advances and challenges in the heterologous production of microbial nitrilases for biocatalytic applications. World J. Microbiol. Biotechnol. 33, 8.

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

Martínková, L., Veselá, A.B., Rinágelová, A., Chmátal, M., 2015. Cyanide hydratases and cyanide dihydratases: emerging tools in the biodegradation and biodetection of cyanide. Appl. Microbiol. Biotechnol. 99, 8875–8882.

Martínková, L., Stolz, A., van Rantwijk, F., D'Antona, N., Brady, D., Otten, L.G., 2014. Nitrile converting enzymes involved in natural and synthetic cascade reactions. In: Riva, S., Fessner, W.D. (Eds), Cascade biocatalysis: integrating stereoselective and environmentally friendly reactions. Wiley-VCH Verlag, Weinheim, pp. 248–270.

McCarty, R.M., Somogyi, A., Lin, G., Jacobsen, N.E., Bandarian, V., 2009. The deazapurine biosynthetic pathway revealed: In vitro enzymatic synthesis of preQ(0) from guanosine-5′-triphosphate in four steps. Biochemistry 48, 3847–3852.

OReilly, C., Turner, P.D., 2003. The nitrilase family of CN hydrolysing enzymes – a comparative study. J. Appl. Microbiol. 95, 1161–1174.

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

Park, J.M., Sewell, B.T., Benedik, M.J., 2017. Cyanide bioremediation: the potential of engineered nitrilases. Appl. Microbiol. Biotechnol. 101, 3029–3042.

Pedras, M.S.C., Minic, Z., Thongbam, P.D., Bhaskar, V., Montaut, S., 2010. Indolyl-3-acetaldoxime dehydratase from the phytopathogenic fungus *Sclerotinia sclerotiorum*: Purification, characterization, and substrate specificity. Phytochemistry 71, 1952–1962.

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

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., 2012b. Purification and characterization of heterologously expressed nitrilases from filamentous fungi. Appl. Microbiol. Biotechnol. 93, 1553–1561.

Piotrowski, M., Schönfelder, S., Weiler, E.W., 2001. The *Arabidopsis thaliana* isogene NIT4 and its orthologs intobacco encode β-cyano-L-alanine hydratase/nitrilase. J. Biol. Chem. 276, 2616–2621.

Piotrowski, M., 2008. Primary or secondary? Versatile nitrilases in plant metabolism. Phytochemistry 69, 2655–2667.

Prasad, S., Bhalla, T.C., 2010. Nitrile hydratases (NHases): At the interface of academia and industry. Biotechnol. Adv. 28, 725–741.

Ramteke, P.W., Maurice, N.G., Babu, J., Wadher, B.J., 2013. Nitrile-converting enzymes: An eco-friendly tool for industrial biocatalysis. Biotechnol. Appl. Biochem. 60, 459–481.

Rustler, S., Chmura, A., Sheldon, R.A., Stolz, A., 2008. Characterisation of the substrate specificity of the nitrile hydrolyzing system of the acidotolerant black yeast Exophiala oligosperma R1. Stud. Mycol 61, 165–174.

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

Sosedov, O., Stolz, A., 2014. Random mutagenesis of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191 and identification of variants, which form increased amounts of mandeloamide from mandelonitrile. Appl. Microbiol. Biotechnol. 98, 1595–1607.

Tao, L., Ma, Z., Xu, X., Bechthold, A., Bian, Y., Shentu, X., Yu, X., 2015. Engineering *Streptomyces diastatochromogenes* 1628 to increase the production of toyocamycin. Eng. Life Sci. 15, 779–787.

Thuku, R.N., Brady, D., Benedik, M.J., Sewell, B.T., 2009. Microbial nitrilases: versatile, spiral forming, industrial enzymes. J. Appl. Microbiol. 106, 703–727.

Vergne-Vaxelaire, C., Bordier, F., Fossey, A., Besnard-Gonnet, M., Debard, A., Mariage, A., Pellouin, V., Perret, A., Petit, J.L., Stam, M., Salanoubat, M., Weissenbach, J., De Berardinis, V., Zaparucha, A., 2013. Nitrilase activity screening on structurally diverse substrates: providing biocatalytic tools for organic synthesis. Adv. Synth. Catal. 355, 1763–1779.

Veselá, A.B., Petříčková, A., Weyrauch, P., Martínková, L., 2013. Heterologous expression, purification and characterization of arylacetonitrilases from *Nectria haematococca* and *Arthroderma benhamiae*. Biocat. Biotrans. 31, 49–56.

Veselá, A.B., Křenková, A., Martínková, L., 2015. Exploring the potential of fungal arylacetonitrilases in mandelic acid synthesis. Mol. Biotechnol. 57, 466–474.

Veselá, A.B., Rucká, L., Kaplan, O., Pelantová, H., Nešvera, J., Pátek, M., Martínková, L., 2016. Bringing nitrilase sequences from databases to life: the search for novel substrate specificities with a focus on dinitriles. Appl. Microbiol. Biotechnol. 100, 2193–2202.

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

Wang, M.X., 2015. Enantioselective biotransformations of nitriles in organic synthesis. Accounts Chem. Res. 48, 602–611.

Wilding, B., Veselá, A.B., Perry, J.J.B., Black, G.W., Zhang, M., Martínková, L., Klempier, N., 2015. An investigation of nitrile transforming enzymes in the chemo-enzymatic synthesis of the taxol sidechain. Org. Biomol. Chem. 13, 7803–7812.

Yeom, S.-J., Lee, J.-K., Oh, D.-K., 2010. A positively charged amino acid at position 129 in nitrilase from *Rhodococcus rhodochrous* ATCC 33278 is an essential residue for the activity with meta-substituted benzonitriles. FEBS Lett. 584, 106–110.

Yusuf, F., Chaubey, A., Jamwal, U., Parshad, R., 2013a. A new isolate from *Fusarium proliferatum* (AUF-2) for efficient nitrilase production. Appl. Biochem. Biotechnol. 171, 1022–1031.

Yusuf, F., Chaubey, A., Raina, A., Jamwal, U., Parshad, R., 2013b. Enhancing nitrilase production from Fusarium proliferatum using response surface methodology. SpringerPlus 2, 290.

Yusuf, F., Rather, I.A., Jamwal, U., Gandhi, S.G., Chaubey, A., 2015. Cloning and functional characterization of nitrilase from *Fusarium proliferatum* AUF-2 for detoxification of nitriles. Funct. Integr. Genom. 15, 413–424.

Zhang, Z.-J., Xu, J.-H., He, Y.-C., Ouyang, L.-M., Liu, Y.-Y., Imanaka, T., 2010. Efficient production of (R)-(−)-mandelic acid with highly substrate/product tolerant and enantioselective nitrilase of recombinant *Alcaligenes* sp. Proc. Biochem. 45, 887–891.

Zhang, Z.-J., Pan, J., Liu, J.-F., Xu, J.-H., He, Y.-C., Liu, Y.-Y., 2011. Significant enhancement of (R)-mandelic acid production by relieving substrate inhibition of recombinant nitrilase in toluene–water biphasic system. J. Biotechnol. 152, 24–29.