



Teze disertace
k získání vědeckého titulu "doktor věd"
ve skupině věd
Biologicko-ekologické vědy

**Microbial Enzyme Transformations of Cyano and Phenolic Compounds:
Advances, Challenges and Perspectives**

název disertace

Komise pro obhajoby doktorských disertací v oboru
Botanika, experimentální a ekologická biologie

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Místo a datum: **Praha, 2. 12. 2016**

Acknowledgements

I would like to express my sincere thanks to all those who participated in the studies underlying this thesis: the members of the Laboratory of Biotransformation, co-operating laboratories at the Institute of Microbiology and at other institutions home and abroad: research fellows, students and technicians. They are mentioned in the dissertation thesis as authors or in the acknowledgements. I would like to thank them for supporting our joint studies with their keen interest, original ideas and extraordinary diligence, for overcoming the difficulties and, importantly, for their friendliness. There are too many of them to mention here.

Nevertheless, I would like to mention at least one of them, the head of the Laboratory of Biotransformation, Professor Vladimír Křen, as this work would never have been completed without the immense efforts he has put into the foundation of the laboratory *ca.* 20 years ago and into its development over the whole period since then. I would like to thank him for his sustained interest in this research topic, for his all-round support, for his fruitful advice and his useful criticism, and for giving me the opportunity of a longstanding research career in his lab.

I would like to thank my late parents and all of my family for supporting me, in all possible ways, during all my studies and my career, and to all my friends, especially Milena and Věra, for their deep understanding and all kinds of support.

I would like to gratefully acknowledge the financial support of this study via grants from the CAS, Czech Science Foundation, Ministry of Education, Youth and Sports, COST *etc.* The relevant projects are mentioned in the dissertation thesis.

Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AMase	Amidase
DBAM	2,6-Dichlorobenzamide
L-DOPA	L-3,4-Dihydroxyphenylalanine
CHT	Cyanide hydratase
CLEAs	Cross-linked enzyme aggregates
CynD	Cyanide dihydratase
IPTG	Isopropyl- β -D-thiogalactopyranoside
na	Not assayed
nd	Not detected
NHase	Nitrile hydratase
NLase	Nitrilase
LAC	Laccase
PPO	Polyphenol oxidase
Ref	Reference
TBBPA	Tetrabromobisphenol A
TBC	<i>tert</i> -Butylcatechol
TYR	Tyrosinase

Summary

Nitrile- and phenol-transforming enzymes are examples of biocatalysts with a wide spectrum of applications in bioremediation and organic synthesis. The aim of this study was to promote their use in biotechnology with a focus on environmental technologies. Therefore, the work encompassed the search for their neglected sources, development of methods for their efficient production and the design of their applications.

Filamentous fungi were examined as a so-far unexploited source of NLases (catalyzing the direct hydrolysis of nitrile into acid). These enzymes were obtained by either culture collection screening or database mining. The latter was followed by artificial gene synthesis and heterologous expression of the gene in *Escherichia coli*. The enzymes belonged to various substrate-specificity subtypes (aromatic NLases, aliphatic NLases or arylacetoNLases). In this way a set of 20 enzymes was created, also including artificial NLase variants. The enzymes were efficient tools for the detoxication of nitrile contaminants and for the synthesis of valuable carboxylic acids.

Rhodococci were also studied as a rich source of not only NLases but also NHases (catalyzing the conversion of nitrile into amide). In these organisms, both enzyme types were active for benzonitrile herbicides (chloroxynil, bromoxynil, ioxynil). It was concluded that these enzymes, mainly the constitutive NHases, may have an important role in the degradation of these herbicides in soil. The metabolites of these compounds (amides, acids) were isolated and their toxicity evaluated. The NLases from rhodococci were also produced recombinantly and their activities for bromoxynil were confirmed in the enzymes thus obtained.

The rhodococcal NHases also proved promising for the degradation of dichlobenil (herbicide) and aliphatic nitrile pollutants, and for the synthesis of amides from nitriles. Together with AMases (catalyzing the acyl transfer from amide to water or other nucleophiles such as hydroxylamine), they were used for the production of carboxylic acids or hydroxamic acids from nitriles. Immobilized NHases (CLEAs) and immobilized whole cell catalysts (LentiKats[®]) were prepared for these purposes.

CHTs (related to NLases, but catalyzing the conversion of cyanide to formamide) were also obtained by database searches. Four of these fungal enzymes were overproduced in *E. coli*. The CHT from *Aspergillus niger* was produced with the highest yield and employed for cyanide detoxication in coal-coking wastewaters.

As part of the aforementioned enzyme screening, innovative assays were developed. Some are based on using new NLase and CHT substrates (dinitriles, 2-cyanopyridine). In addition, a fast NHase screening was based on a colorimetric reaction (AMase-catalyzed reaction of amide with hydroxylamine, followed by forming a color complex).

PPOs (LACs or TYRs catalyzing the oxidation of phenols and diphenols) were obtained by similar strategies to the above enzymes (culture screening, database searches). LACs were applied in the efficient degradation of brominated phenols and TBBPA (flame retardant).

Compounds identical to mammalian metabolites of the latter compound were prepared with LACs for biological studies.

A new TYR from *Polyporus arcularius* was produced in *E. coli* in its latent form, and the enzyme was activated by a partial proteolytic cleavage *in vitro*. This method proved to give high TYR yields. The enzyme was suitable for testing the inhibitors of melanogenesis.

Using the TYR isolated from fruiting bodies of *Agaricus bisporus* (common button mushroom), methods were proposed for the degradation of phenolic compounds (phenol, cresols) in industrial wastewaters. The TYR was combined with CHT in a two-step process. Cyanide (inhibitor of TYR) was enzymatically removed in the first step and TYR was applied to the cyanide-free medium in the second. This approach is promising for the treatment of heavily polluted coal-coking wastewaters by cyanide-sensitive oxidases in general.

The library of enzymes and the methodology created in this work (rational database mining based on structure-activity relationships, optimized expression of artificial genes, enzyme purification and assays) provide conditions for further studies towards the development of enzyme-based nitrile, cyanide and phenol transformations.

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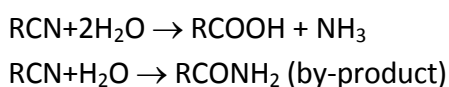
Note: Citations **1-23** refer to the “List of selected publications”. Citations [1]-[39] refer to part 5 (“References”).

1 Background - the enzymes under study

In the last century, enzymes were recognized as highly efficient tools for the transformation of non-natural compounds [1]. This finding was utilized in various branches of biotechnology. The use of enzymes in this area has significantly increased since the beginning of this millennium due to progress in gene and protein studies.

The use of enzymes in environmental technologies is an emerging application with significant advantages over using cell cultures (sensitive) or chemical agents (toxic or harmful). Various microbial hydrolases or oxidases have great potential in this area. In this thesis, two groups of enzymes – i) nitrile- and cyanide converting enzymes and ii) PPOs – have been under study. The focus was on their application in the detoxication of cyanide/nitrile and phenolic pollutants, respectively. Attention has also been paid to their emerging uses for the preparation of fine chemicals and metabolites of xenobiotics, and as models for inhibition studies.

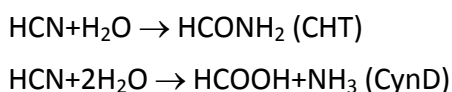
The nitrile-converting enzymes consist of NLases and NHases, which are evolutionarily unrelated. NLases hydrolyze their substrates (aliphatic or aromatic nitriles, arylacetoneitriles) into carboxylic acids, often with the formation of various amounts of amide as by-product (Scheme 1).



Scheme 1 Reactions catalyzed by NLases

The catalytic triade E-K-C is conserved in all proteins of the NLase superfamily [2]. NLases are classified into subtypes according to their substrate specificities for aliphatic, aromatic nitriles and arylacetoneitriles. Their biotechnological potential is based on their broad substrate specificities and mild reaction conditions. In addition, many of them are regio- and enantioselective [1] [3].

Cyanide-converting enzymes are specific subtypes of NLases with a strong preference for free cyanide. They are comprised of CHTs and CynDs, which convert cyanide into formamide or formic acid, respectively (Scheme 2). Their potential is in the detoxication of cyanide [2] [3].



Scheme 2 Reactions catalyzed by CHTs and CynDs

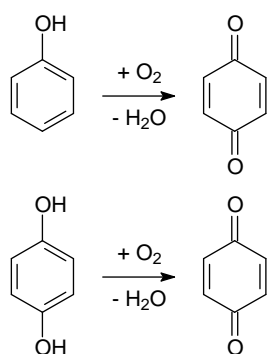
NHase is a metalloenzyme with a non-heme Fe^{3+} or a non-corrinoid Co^{3+} cofactor [4]. Amides are the only products of NHases, but they are often hydrolyzed by AMases produced by the same organism (Scheme 3). Hence, the products and uses of the NHase/AMase enzyme systems are similar to those of NLases [5].



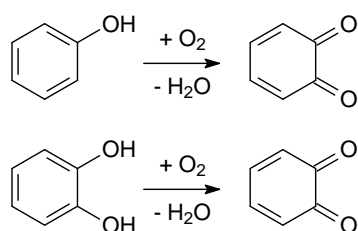
Scheme 3 Reactions catalyzed by NHases and AMases

The natural role of nitrile-converting enzymes seems to reside in the turnover of nitriles such as β -cyanoalanine (intermediate of HCN assimilation), cyanogenic glycosides or indoleacetonitrile [6].

PPOs are comprised of LACs and TYRs, both of which are multicopper enzymes but differ in their preferred substrates: 1,4-diphenols for LACs (Scheme 4) and 1,2-diphenols for TYRs (Scheme 5) [7]. Their primary products are free radicals, which then give rise to quinones or to oligo- or polymeric substances. These enzymes are highly attractive for biotechnology due to their ability to utilize molecular oxygen and their wide substrate specificities. The uses of PPOs include the elimination of phenolic pollutants, food processing, biocatalysis and melanogenesis inhibition tests [19](#).



Scheme 4 Reactions catalyzed by LACs



Scheme 5 Reactions catalyzed by TYRs

PPOs are involved in some key events of cell differentiation, cell defence, stress response and virulence. LACs, which are abundant in fungi, plants and prokaryotes, participate in the sclerotization of insect cuticles, formation of the UV-resistant spores in bacilli, formation of plant cell walls, protection of fungal pathogens against some plant defence compounds

(phytoalexins, tannins), and degrade lignin in white-rot fungi [8]. TYRs catalyze the formation of melanin and melanin-like pigments in animals, plants and microorganisms [9]. Their improper functioning may cause skin pigmentation disorders in humans [10].

2 Nitrile- and cyanide-transforming enzymes

2.1 Enzyme sources and production methods

Bacterial and plant NLases have been reported since the 1960s, and their roles in the detoxication and recycling of natural nitriles were hypothesized. The occurrence of NLases in filamentous fungi was largely neglected, and only two fungal NLases (in genus *Fusarium*) were characterized until 1989 [11]. In the 1980s an alternative nitrile-hydrolysis pathway was found in bacteria, which was comprised of NHase and AMase [4]. Culture collection screens or the selection of bacteria from natural sources such as soils were initially the only methods to obtain the organisms with nitrile-transforming activities. Later, the cloning of the relevant genes enabled more of the enzymes to be obtained via advanced methods such as metagenome mining and database searching.

2.2.1 Production in wild-type strains

The production of NLases in some wild-type bacteria is highly efficient due to the high expression levels of the relevant genes in the presence of a strong inducer [11]. For instance, isobutyronitrile was found to induce NLases in various rhodococci [12]. This enabled NLases to be obtained for the biodegradation of benzonitrile herbicides (see part 2.3.1), which were not NLase inducers *per se*.

NHases may be inducible or constitutive. In *Rhodococcus* sp. J1, the strain used in the industrial production of acrylamide, two different Co-type NHases (low- or high-molecular weight NHase) were produced, depending on the inducer (cyclohexanecarboxamide or urea, respectively) [4]. In some strains of rhodococci, including *Rhodococcus erythropolis* A4 which we used, the production of the Fe-type NHases was constitutive [14]. The organism was an alternative catalyst for the transformation of benzonitrile herbicides (see part 2.2.1). This strain or its purified NHase also proved to be efficient catalysts for the preparation of amides from various nitrile substrates such as 3-oxo-, 3-hydroxy and 4-hydroxy nitriles [15, 16].

The first fungal NLase was purified from *Fusarium solani* in 1977 but the amino acid sequence of the enzyme remained unknown (for a review, see [11]). The finding that the subunit mass in this enzyme was approximately 76 kDa is surprising, the usual masses of typical NLases being approx. 40 kDa. In our work, the same strain gave a typical NLase composed of 40 kDa subunits. In this experiment, the fungus was grown with 2-cyanopyridine as a strong inducer [5], which resulted in high NLase yields. A partial amino acid sequence of the purified enzyme was obtained using mass spectral measurements [6]. A hypothetical protein with a high similarity to this NLase was found in GeneBank. The amino acid sequence of this hypothetical NLase was deduced from the gene sequenced in *Gibberella*

moniliformis. This gene was later expressed in *E. coli* (see part 2.2.2). The corresponding protein product was demonstrated to have a similar substrate specificity to the aforementioned NLase from *F. solani*, both enzymes acting preferentially on aromatic nitriles [6, 8].

Similar NLases were also produced by other members of *Ascomycota*, such as *Aspergillus niger*, *Fusarium oxysporum* or *Penicillium multicolor* [3-5]. The hyperinduction strategy seems to be widely applicable for the production of aromatic NLases in fungi [5, 6] and yeast (*Exophiala oligosperma* [12]). However, it was not possible to obtain NLases with other substrate specificities (such as arylacetoNLases) in this way. An alternative screening strategy based on database searches had to be used for this purpose (see part 2.2.2).

2.2.2 Heterologous production

NLases from various bacterial sources have been previously produced in recombinant strains of *E. coli* under standard conditions (lysogeny broth (LB), inducer IPTG; e.g., [13-15]). Some of the production methods were optimized and scaled up (to a maximum of 300 L). In this case, IPTG was replaced with a cheaper inducer, lactose [16]. Using autoinduction medium or induction with rhamnose was also found to be a useful alternative to IPTG induction [17, 18].

The heterologous production of aromatic NLases and arylacetoNLases from fungi was first examined by our group. These enzymes were produced in *E. coli* under similar conditions to the bacterial NLases. The activity yields were then optimized by varying several cultivation parameters. The commercial medium EnPresso[®] was superior to LB or yeast extract – tryptone (YT) media in terms of the culture density and NLase yield achieved. It was also advantageous to decrease the IPTG concentrations to 0.02 mM in order to avoid excessive translation rates, which may lead to protein misfolding [9]. Correct folding of the enzymes was also supported by the presence of some chaperones. A set of commercial plasmids encoding various bacterial chaperones was used, and the GroEL/ES chaperones were found to increase the NLase yields significantly. This effect was enzyme-specific: positive effects of this chaperone coexpression were observed in the aromatic NLases from *Gibberella moniliformis* and *Penicillium marneffeii*, but not in arylacetoNLases [19].

The effect of the *E. coli* strain was also examined. Both *E. coli* BL21 and Origami B gave active NLases, but the enzyme yields were higher in the latter strain. This was probably due to the different environments for protein folding; the latter strain supports the formation of disulfide bonds in the recombinant protein [9].

While only a few aromatic NLases have been successfully produced in wild-type fungi (see part 2.2.1), the production of NLases in *E. coli* hosts enabled us to obtain an array of NLases of various substrate-specificity types (aromatic and aliphatic NLases, arylacetoNLases, a NLase with a broad substrate-specificity) and CHTs [8, 9]. In this way, a library of NLases of different origins and with different catalytic properties was obtained, which also contained four bacterial and a plant NLase to complete the set (Table 1). Sequences indicative of the

substrate specificities of the enzymes were found in the regions surrounding the catalytic cystein **8**.

All the NLases examined in this study acted on various dinitriles as substrates, albeit with varying rates (Table 2). Fumaronitrile was a good substrate for all the enzymes examined, including CHTs. Thus this dinitrile may be used in CHT assays instead of cyanide (highly toxic, unstable). Phenylenediacetonitriles and cyanophenylacetoneitriles were excellent substrates of all arylacetoNLases examined, and some of them were also substrates of other NLase types. These findings indicate that some dinitriles may be useful as substrates for NLase assays and screens. Furthermore, the NLases preferentially hydrolyzed one cyano group in the dinitriles examined **9**. Therefore, they may be used to obtain the valuable cyano acids or cyano amides (building blocks) from these substrates.

The site-directed mutagenesis of NLases focused on the region surrounding the active cystein. This region was important for the activity of the enzyme, its tendency to produce amide (by-product) and its enantioselectivity for mandelonitrile [17, 20]. Similar substitutions produced similar effects in fungal and bacterial NLases despite the low degree of identity of the proteins, which was *ca.* 40%. In this way, NLases able to produce a high amide : acid ratio were obtained, which may be used instead of an NHase (unstable, poorly enantioselective) to transform nitriles into amides. For instance, NLase from *Neurospora crassa* produced mandelamide at 85% of the total product, i.e. almost six times more than the wild-type NLase **10**.

Table 1 “Library” of NLases and CHTs created in this study **3, 6, 8-10**

NLase type	Organism	Protein accession no.	Preferential substrate ^a
Aromatic NLase	<i>Aspergillus kawachii</i>	dbj GAA90167.1 ^b	Benzonitrile; 4-Cyanopyridine
	<i>Gibberella moniliformis</i>	gb ABF83489.1 ^c	
	<i>Meyerozyma guilliermondi</i>	ref XP_001482890.1	
	<i>Penicillium marneffeii</i>	ref XP_002144951.1	
	<i>Penicillium chrysogenum</i>	ref XP_002565836.1	
	<i>Rhodococcus rhodochrous</i>	emb CCN27134.1	
	<i>Rhodococcus ruber</i>	emb CCN27135.1 emb CCN27136.1	
Aliphatic NLase (Plant type)	<i>Sinorhizobium fredii</i>	ref YP_005193111.1	Phenylacetoneitrile
	<i>Sorghum bicolor</i>	ref XP_002452498.1	
ArylacetoNLase	<i>Aspergillus kawachii</i>	dbj GAA83217.1	Phenylacetoneitrile; Mandeloneitrile
	<i>Aspergillus niger</i>	ref XP_001397369.1 ^d ref XP_001398633.1	
	<i>Aspergillus oryzae</i>	ref XP_001824712.1	
	<i>Arthroderma benhamiae</i>	ref XP_003011330.1	
	<i>Auricularia delicata</i>	gb EJD42068.1	
	<i>Nectria haematococca</i>	ref XP_003050920.1	
	<i>Macrophomina phaseolina</i>	gb EKG14506.1	
	<i>Neurospora crassa</i>	emb CAD70472.1 ^d	
Broad substrate- specificity NLase	<i>Trichoderma virens</i>	gb EHK18468.1	Phenylacetoneitrile; Benzonitrile
CHT	<i>Aspergillus niger</i>	gb ABX75546.1	HCN
	<i>Botryotinia fuckeliana</i>	ref XP_001551197.1	
	<i>Penicillium chrysogenum</i>	ref XP_002562104.1	
	<i>Pyrenophora teres f.teres</i>	ref XP_003301539.1	

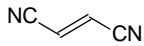
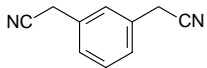
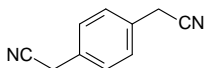
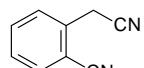
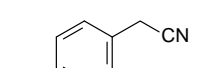
^aof the mononitriles examined

^ba similar NLase was obtained from wild-type *Aspergillus niger*

^ctwo similar NLases were obtained from wild-type *Fusarium solani*

^dmutants created by site-directed mutagenesis

Table 2 Activities of different NLase subtypes for dinitriles **9**

Substrate	Activity (U mg ⁻¹ of protein)						
	Aromatic NLase	ArylacetoNLases			Broad substrate specificity NLase	Aliphatic (plant-type) NLase	CHT
		<i>Aspergillus</i>	<i>Auricularia</i>	<i>Macrophomina</i>	<i>Trichoderma</i>	<i>Sorghum</i>	
		<i>kawachii</i>	<i>delicata</i>	<i>phaseolina</i>	<i>virens</i>	<i>bicolor</i>	
	39	2.9	9.9	7.9	5.3	0.87	
	0.75	64	241	38	16	nd	
	0.27	17	44	8.9	5.1	nd	
	0.38	16	59	12	2.3	nd	
	0.70	43	159	21	7.4	0.22	

The best substrate for each NLase is marked in bold.

2.3 Enzyme applications

This part of the study focused on the biodegradation pathways of benzonitrile herbicides (see part 2.3.1) and the biodegradation of free cyanide (see part 2.3.2). Other possible uses of the aforementioned nitrile-converting enzymes are e.g. the detoxication of aliphatic nitriles (acetonitrile, propionitrile or butyronitrile used as industrial solvents). Some of the fungal NLases (both wild-type and recombinant) and the bacterial NHase/AMase system exhibited significant activities for these substrates [6, 8, 11].

Bacterial and fungal cells may be immobilized for these purposes. An industrially viable immobilization method is e.g. entrapment in lens-shaped particles made of polyvinylalcohol and polyethylene glycol (LentiKats®). This approach has been examined for a variety of enzyme applications. It also proved to be useful for the immobilization of *Rhodococcus* cells with NHase/AMase activities [15] and *F. solani* mycelium with NLase activity [21].

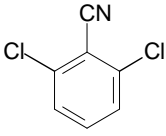
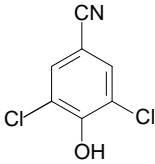
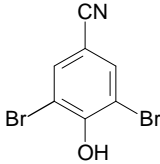
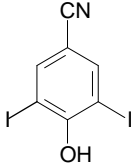
2.3.1 Biodegradation of benzonitrile herbicides

Benzonitrile herbicides are halogenated derivatives of benzonitrile and consist of two types – 3,5-dihalogeno-4-hydroxybenzonitriles (selective against broad-leaved weeds) and 2,6-dichlorobenzonitrile (dichlobenil; non-selective). A widely used compound of the first type is bromoxynil. In contrast, dichlobenil was banned in the EU in 2008 (EC regulation No. 689/2008). Nevertheless, the pollution of the environment with its metabolite DBAM persists (for a review, see [22]).

The ability of various bacteria to degrade benzonitrile herbicides was reported [22], but little attention was paid to rhodococci. These organisms are widespread in soil, and hence they may be crucial for the breakdown of these compounds in this environment [11]. They exhibit both NHase and NLase activities which may act on the cyano group in benzonitrile derivatives.

The transformation of dichlobenil to the corresponding amide has been reported for a number of NHases [22]. Here, the products of benzonitrile herbicides were examined in two types of rhodococcal NHases – the Fe-type NHase in *Rhodococcus erythropolis* (constitutive) and Co-type NHase in *Rhodococcus rhodochrous* (inducible) (Table 3). The former NHase was much more active for dichlobenil than the latter. Furthermore, the product of this reaction, DBAM, was partly hydrolyzed by *R. erythropolis* into the corresponding acid after prolonged reaction times [14]. Previously this activity was found in *Aminobacter* sp. but produced only low amounts of the acid product [23]. No activity of NLases for dichlobenil was found.

Table 3 Transformation of benzonitrile herbicides by *Rhodococcus erythropolis* with NHase activity and *Rhodococcus rhodochrous* with NHase or NLase activity [12, 14]

Organism, enzyme	Time (h)	Conversion (%) ^a							
									
		Amide	Acid	Amide	Acid	Amide	Acid	Amide	Acid
<i>R. erythropolis</i> , NHase (Fe-type)	24	65 ^b	26	nd ^b	88	33 ^b	67	47	3
<i>R. rhodochrous</i> , NHase (Co-type)		27	nd	96	4	95	1	92	2
<i>R. rhodochrous</i> (NLase)	5	nd	nd	nd	99	nd	86	nd	41

^aDetermined by HPLC. Conditions: 0.5 mM substrate, 10 mg dry cell weight mL⁻¹ (NHase) or 0.4 mg dry cell weight mL⁻¹ (NLase); 30 °C.

^bAlmost total conversion into amide was achieved after shorter reaction times (30 min, 1 h or 3 h for dichlobenil, chloroxynil and bromoxynil, respectively). Amide was then hydrolyzed further into acid.

In contrast to dichlobenil, bromoxynil and analogues were transformed by both NHases and NLases. Both Fe- and Co-type NHase transformed these compounds smoothly, but the former exhibited higher activities. In addition, *R. erythropolis* (but not *R. rhodochrous*) hydrolyzed the amide intermediates into significant amounts of acids [14].

The highest NLase activity for bromoxynil and analogues was previously found in *Klebsiella pneumoniae* subsp. *ozaenae*, and the corresponding gene was used to construct transgenic plants [24]. However, the knowledge of the activity of rhodococcal NLases towards these compounds was marginal [11, 22]. Here, all three examined strains hydrolyzed these compounds smoothly into acids (Table 3) [12]. In both the NHase and NLase, the reaction rates decreased with the increasing bulkiness of the substrate, i.e. from chloroxynil to ioxynil. A similar effect was observed for the hydrolysis of amides by AMase in *R. erythropolis* [14].

The amide and acid products of all the parent compounds were obtained by appropriate enzymatic reactions (amides by NHase, acids by NLase or NHase + AMase) and purified. The expected structures were confirmed by spectral data (NMR, MS) [12, 14].

All products were examined for their acute toxicities using two model organisms: *Vibrio fischeri* and *Lactuca sativa* (garden lettuce). The tests with the first model, in which the effect of the compounds on its luminiscence was monitored, led to the following findings: Two amides (from chloroxynil and dichlobenil) were less toxic than the parent nitriles. the

other two (from bromoxynil and ioxynil) were slightly more toxic. In contrast, the acids from chloroxynil and bromoxynil were less toxic, but those from ioxynil and especially dichlobenil were more toxic than the corresponding nitriles. In *L. sativa*, the inhibition of seed germination was the highest with nitriles. The amides and acids retained between 42% and 93% of the inhibition potential of the nitriles [12, 14].

In conclusion, the hydration or hydrolysis of the cyano group of benzonitrile herbicides did not lead to the detoxification of these compounds. However, it can be the initial step in their breakdown and, ultimately, mineralization. It is likely that rhodococci have an important role in the degradation of these compounds in soil. Especially *R. erythropolis*, with its constitutive NHase/AMase pathway, is likely to be an important agent in their transformation.

2.3.2 Biodegradation of cyanide

The use of enzymes such as CynDs and CHTs represents a new, promising option in cyanide degradation due to their advantages over living cells (sensitive) and chemical agents such as chlorine or hypochlorite (toxic, giving rise to toxic by-products) [23]. The focus of this study was on recombinantly produced CHTs. Their application potential was examined with model and real wastewaters.

A few CHTs from wild-type fungi [11] and four recombinant CHTs [25] were reported prior to our study. In this study, a further four CHTs became available by heterologous production [8, 9, 13]. These CHTs were obtained by expressing the genes from *Aspergillus niger*, *Botryotinia fuckeliana*, *Penicillium chrysogenum* and *Pyrenophora teres* f. *teres* (Table 1). In these organisms, CHTs were reported for the first time.

All the recombinant proteins were produced in *E. coli* with high yields and exhibited high specific activities for HCN (≥ 100 U mg⁻¹ protein). The CHT from *A. niger* was produced at the highest levels, forming approximately one half of the total soluble protein. Its specific activity was also the highest (ca. $1.3 \cdot 10^3$ U mg⁻¹ protein; V_{\max} $6.8 \cdot 10^3$ U mg⁻¹ protein).

Activities for substrates other than HCN were found in all these CHTs. The highest were determined with fumaronitrile and 2-cyanopyridine (Table 4), which therefore could serve as substrates in CHT assays instead of HCN. The kinetics of these substrates was determined in CHT for the first time. The CHT from *A. niger* was used for this purpose. Both V_{\max} and K_m were lower for its nitrile substrates compared to HCN (Table 4). The lower K_m for nitriles corroborates the hypothesis that CHTs evolved from NLases. Their genes could have been acquired by fungi by horizontal transfer from bacteria. The enzymes could have then been adapted to obtain activity for HCN [26].

Table 4 Substrate specificity of CHT from *Aspergillus niger* **13**

Substrate	Specific activity ^a (U mg ⁻¹ protein)	V _{max} (U mg ⁻¹ protein)	K _m (mM)
HCN	1.3 · 10 ³	6.8 · 10 ³	109
Benzonitrile	0.82	na	na
2-Cyanopyridine	10.1	10.3	3.7
3-Cyanopyridine	0.72	na	na
Fumaronitrile	12.6	18.8	14.7

^awith 25 mM of substrate

The ability of CHT to degrade high concentrations (25 mM) of HCN was demonstrated using a continuous reactor loaded with *E. coli* cells containing the enzyme from *A. niger*. The catalyst was held in place by a semipermeable cellulose membrane. No cyanide could be detected at the outlet for the 3 days of operation. Formamide was detected as the only reaction product **13**. This compound is much less toxic than HCN; in addition, it is likely to be further degraded at the sewage disposal plant.

Moreover, this CHT was able to remove HCN from real samples of coking wastewaters. This type of wastewater contains large amounts of cyanide, phenolic compounds and salts (sulfide, thiocyanate *etc.*). Neither phenols nor salts were inhibitory to CHT to a significant extent. The typical concentration of HCN in these wastewaters was *ca.* 0.6 mM, and decreased almost to zero after treatment with CHT. Moreover, the enzyme was also able to eliminate HCN from spiked samples with 10-20 mM HCN (mimicking shock loads) **23**.

The enzymatic removal of HCN also increased the efficiency of TYR used to degrade phenols in these wastewaters. This enzyme is strongly inhibited by cyanide. The elimination of HCN alleviated this inhibition and enabled us to better exploit its biodegradation potential (see part 3.2.3).

3 Polyphenol oxidases

3.1 Production in wild-type and recombinant strains

Common sources of PPOs are fungi of the phylum *Basidiomycota* (fruiting bodies or mycelia). The LAC from *Trametes versicolor* and the TYR from *Agaricus bisporus* have been most intensively studied due to their commercial availability [19]. The heterologous production of PPOs has been used less frequently. This is probably because i) the wild-type fungi are excellent sources of PPOs, ii) PPOs require posttranslational modifications, and iii) they may be toxic to the host. In this study, LACs were obtained from wild-type sources, while both production strategies were examined with TYRs.

3.1.1 Laccase production

An intensive screening of the in-house collection of basidiomycetous fungi (Culture Collection of *Basidiomycota* - CCBAS, Institute of Microbiology) led to finding several LAC producers able to degrade brominated (bis)phenols. These fungi belonged to the species *Trametes versicolor*, *Trametes pubescens*, *Trametes villosa*, *Trametes gibbosa*, *Pleurotus ostreatus*, *Dichomitus squalens* and *Bjerkandera adusta*. The strains were grown in submerged mode using 3,4-dimethoxybenzyl alcohol as the LAC inducer. Their degradation abilities were largely in correlation with their LAC activities. *T. versicolor* was the best LAC producer and brominated-phenol degrader. Its purified LAC (commercial) was used to prepare the metabolites of TBBPA and brominated phenols [20, 21].

3.1.2 Tyrosinase production

In this study [22], TYR was extracted from the common button mushroom (*Agaricus bisporus*). A commercial TYR preparation from the same source was also used. The former preparation (crude TYR) could be obtained in bulk quantities at low costs. Despite its lower specific activity compared to the commercial preparation (purified), it was suitable for application studies such as the remediation of coking wastewaters (part 4.2.3).

The recombinant production strategy was examined with a new TYR from *Polyporus arcularius* [22]. TYR activity was previously found in the wild-type strain expressing the relevant gene, but the enzyme was not characterized [27]. In our study, the feasibility of expressing the gene in *E. coli* was examined. The production of TYRs in prokaryotic hosts is rarely used [28, 29]. This is probably because TYRs are produced in their latent forms in these hosts, which makes it necessary to activate the isolated enzyme *in vitro*. However, the production of latent TYR may turn out to be an advantage, as this form of the enzyme is not toxic to the host. Thus it may be possible to produce TYRs in large quantities in this way. The activation of the enzyme is simple, consisting of the treatment of the latent TYR with a protease such as trypsin or α -chymotrypsin [22] [28, 29]. Previously, *E. coli* was examined as the host for the production of the latent TYR from the basidiomycete *Pholiota microspora*.

This method was found to be feasible, but the yield of the recombinant enzyme was not reported [28].

In our study [22], the latent TYR from *P. arcularius* was produced in large amounts in *E. coli* under optimum conditions (low IPTG concentration (0.02 mM), low temperature (20 °C), with the coexpression of GroEL/ES chaperones *etc.*). After activating the purified latent TYR with trypsin, an excellent activity of *ca.* 9,000 U L⁻¹ (with *tert*-butylcatechol as substrate) was achieved. The active enzyme was a dimer with a molecular mass of *ca.* 79 kDa. Apart from TBC, it also oxidized L-DOPA, L-tyrosine, phenol and *p*-cresol with relative activities of 42%, 1%, 4% and 37% compared to TBC. It was fairly stable at elevated temperatures of up to 55 °C and exhibited its optimum activity at 70 °C.

The activation of this TYR resided in a partial proteolytic cleavage, resulting in the removal of its C-terminal part, which probably prevents the substrate from accessing the active site. The activation is similar in the fungal and plant TYRs examined [30]. The linker between the N-terminal part carrying the active site and the C-terminal part is susceptible to proteolytic cleavage. It is probably not necessary to cleave the linker after a specific residue: The latent TYR from *Trichoderma reesei* was cleaved at different sites depending on the host - after G400 in the native host [31] and after R407 in *Pichia pastoris* [32]. Both resulting enzymes were active, although they exhibited some differences in their specific activities and pH profiles. The cleavage site depends on the substrate specificity of the protease used; thus trypsin used in our study cleaved the latent TYR after R388, *i.e.* in the linker region. This site may not be the same as in the native host.

3.2 Enzyme applications

This study was focused on the ability of PPOs to transform some common phenolic environmental contaminants (bromophenols – industrial chemicals, herbicides; phenol and cresols – by-products of coal-coking; TBBPA – flame retardant). Possible uses of various types and forms of the enzymes were suggested (crude LACs and whole cells for the degradation of bromophenols and TBBPA; crude TYR for the degradation of phenol and cresols, purified LAC for the preparation of TBBPA metabolites for biological studies). In addition, the utility of recombinant TYR as a model for inhibition studies was demonstrated.

3.2.1 Biodegradation of brominated phenols

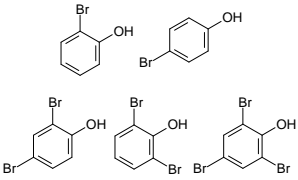
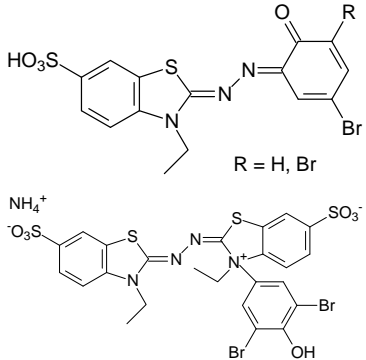
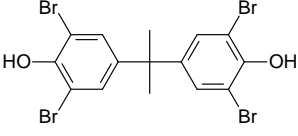
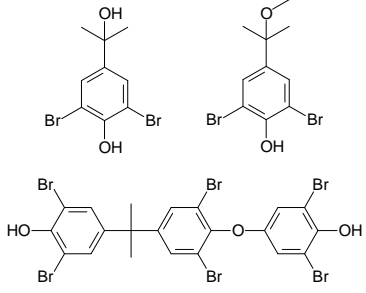
Brominated phenols occur in the environment as a result of human activities (industry, agriculture, transport) but also as natural products. For instance, 2,4,6-tribromophenol (TBP) has been used as a flame retardant or pesticide, and it is also formed by the combustion of leaded fuels together with some mono- and dibromophenols. The latter compounds are also naturally produced in a marine environment [33].

Although LACs have been intensively examined as tools for the degradation of phenolics [19], their potential to degrade brominated phenols was largely neglected. In our study, LAC from *T. versicolor* or the cultures of this organism were found to be suitable for the degradation of various compounds of this type (Table 5). Cell cultures degraded all the examined compounds, albeit at varying rates depending on the substitution pattern. However, the purified LAC from this organism was less efficient, and the addition of ABTS was required for the degradation of bromophenols. A new type of product was isolated from the reaction mixtures – adducts of the brominated phenols and ABTS (Table 5) [20].

3.2.2 Biodegradation of tetrabromobisphenol A

TBBPA is produced in bulk and added to a wide range of industrial products (computers, cars, textiles *etc.*) to improve their fire safety. Few works have addressed its potential microbial degradation. The initial step in its degradation in soil was found to be its anaerobic debromination, as with TBP [34, 35]. TBBPA is widespread in the environment and recalcitrant due to its low solubility and high resistance to microbial attack. However, LACs proved to be efficient degraders of it in our studies [20, 21]. Various cell cultures of basidiomycetous fungi (see also part 4.1.1) were able to degrade this compound (Table 5), the best degrader being *T. versicolor*. No reaction products were found in these cultures, but some were identified when using the purified LAC (Table 5). These were two monoaromatic compounds (one of them identical to a mammalian metabolite of TBBPA) and a triaromatic compound (also found as a mammalian product of TBBPA transformation). Therefore, the use of LAC provided a simple way to obtain sufficient amounts of these metabolites, which were previously prepared with human or rat liver fractions [36]. Estrogenic effects were found in the monoaromatic products via the tests performed with human breast cancer cells carrying estrogenic receptors [21].

Table 5 Biotransformation of brominated phenolics by PPOs **20, 21**.

Compound (mg L ⁻¹)	Catalyst	Degradation (%)	Product(s)
 <p>Mono-, di-, and tribromophenols (420-820)</p>	LAC (commercial)	40-100 ^a	 <p>R = H, Br</p> <p>Products from 4-bromophenol and 2,4- and 2,6-dibromophenol^b</p>
<p>Mono-, di-, and tribromophenols (172-328)</p>	<i>Trametes versicolor</i> ^c	60-80 ^a	nd
 <p>TBBPA (1350)</p>	LAC (commercial)	100	
<p>TBBPA (540)</p>	<i>Trametes versicolor</i> ^c	85	nd

^adepending on the substitution pattern

^bwith ABTS

^ckey enzyme LAC

3.2.3 Bioremediation of phenol-containing wastewaters

Wastewaters with a significant content of phenolics originate from various industrial and agricultural production processes. Many of these compounds are known for their deleterious effects on ecosystems or human and animal health. PPOs are among the most extensively examined tools for the biodegradation of these pollutants. Thus LACs were shown to degrade phenol derivatives in the wastewaters from various food industries such as olive oil production, wine distillation or meat smoking [19].

The focus of our study [23] was on the wastewaters from coal coking. These wastewaters are heavily polluted, containing phenol, cresols, and various other contaminants (polyaromatic substances, cyanide, thiocyanates, sulfides *etc.*). Previously, an immobilized LAC was found to be effective for the elimination of phenols and cresols (total content *ca.* 0.45 g L⁻¹) from this type of wastewater [37].

In our work, TYR was first examined as a potential tool for the treatment of coking wastewater. The samples we used contained more phenol and cresols than the one above (over 2 g L⁻¹ in total) and significant amounts of cyanide (up to *ca.* 15 mg L⁻¹, i. e. *ca.* 0.6 mM). TYR was able to degrade high concentrations of phenol in the model mixtures without cyanide. However, cyanide decreased its phenol-degrading activity by *ca.* 40% at 0.1 mM and almost totally at 1 mM. This problem was solved by removing cyanide prior to the application of TYR. The CHT prepared in *E. coli* as described above (see part 2.3.2) was used for this purpose. This enzyme was almost fully active in the coking wastewater and decreased the cyanide content in this environment to almost zero. The reaction product formamide was not deleterious to TYR.

The consecutive application of TYR decreased the concentration of phenolics (phenol, cresol) to less than 0.2 g L⁻¹. This two-step process was compared to single-step ones (employing only PPOs) in terms of the degradation percentage and the removal rate (Table 6). LAC or *T. versicolor* cell cultures degraded 99-100%, and TYRs 91-94% of the phenolics, which was probably due to the poor activity of TYR for *o*-cresol. However, it must be noted that the concentration of phenols was different in different samples. The highest removal rate of 425 mg of phenolics L⁻¹ h⁻¹ was calculated in our study, followed by 148 and 126 mg of phenolics L⁻¹ h⁻¹ with immobilized LAC and whole cells of *T. versicolor*, respectively (Table 6).

The bienzymatic process provides a new solution for the remediation of heavily polluted wastewaters from coking and similar industries (e.g. coal gasification). The enzymes used are environmentally benign and available in large quantities. The heterologous production of CHT in *E. coli* has been solved, and TYR can be readily obtained from commercial mushrooms at reasonable costs. Similar two-step processes can probably be performed with other metalloenzymes sensitive to cyanide (LACs, peroxidases). Scaling up the process will require making the catalyst utilizable in repeated or continuous mode. Studies are underway to optimize the form of the catalyst for this purpose by immobilization.

Table 6 Removal of phenolics from coal coking/gasification wastewaters - comparison of processes catalyzed by TYR or LAC

Compound (g L ⁻¹)	Catalyst	Degradation (%)	Maximum removal rate [mg L ⁻¹ h ⁻¹] ^a	Ref
Phenol and derivatives (ca. 0.45 total) ^a	Immobilized LAC	99	148	[37]
Phenol and derivatives (0.40 total) ^a	TYR	94	16	[38]
Phenol, cresols (ca. 3.6 total) ^b	<i>Trametes versicolor</i> ^c	100	126	[39]
Phenol, cresols (ca. 2.1 total) ^a	CHT + TYR	91	425	23

^acoal coking wastewater

^bcoal gasification wastewater

^ckey enzyme LAC

4 Conclusions and future outlook

Two groups of industrially important enzymes (nitrile- and cyanide-converting enzymes, PPOs) were studied, aiming to improve their exploitation in bioremediation and biotransformation. New sources of the enzymes were found, especially among the members of *Ascomycota* (for NLases) and *Basidiomycota* (for LACs and TYRs). For instance, the number of characterized fungal NLases was increased from three to over twenty in this study. Methods were developed for obtaining these enzymes by isolation from wild-type strains and by production in heterologous hosts. The latter were based on database searches followed by gene synthesis and expression, and the characterization of the purified proteins. Our understanding of the structure-activity relationships in the enzymes was improved and will simplify further searches for appropriate enzymes. The application potential of the new enzymes was demonstrated to be broad, including the detoxication of nitrile contaminants, detoxication of cyanide and elimination of toxic phenolics in wastewaters, transformation of xenobiotics into their metabolites for toxicity studies, the industrial use of biocatalysts and inhibitor testing. The materials obtained (strains, enzymes) and methods developed have the potential to promote further studies of the aforementioned enzymes and support their uses at the lab and industrial scale.

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6 List of publications included in the dissertation thesis

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