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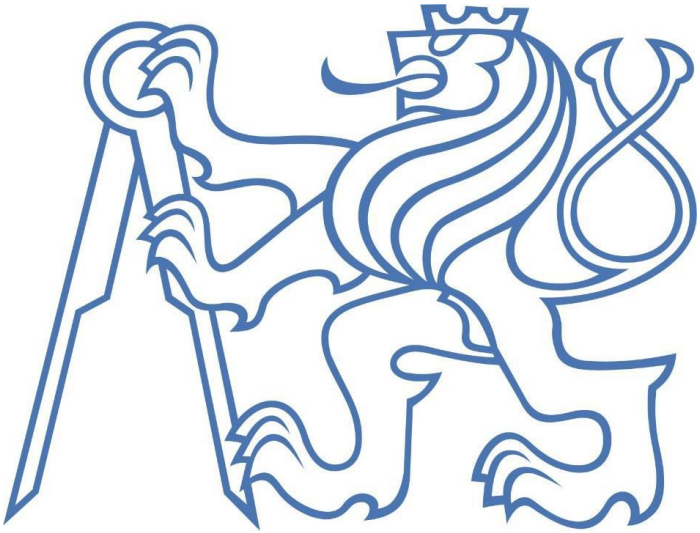

Doctoral Thesis Statement

CZECH TECHNICAL UNIVERSITY IN PRAGUE
Faculty of Biomedical Engineering
Department of Health Care Disciplines and Population Protection

Anastasia Sedova

Efficient bioremediation of toxic waste by new nitrilases

Ph.D. Program: P1032 Civil Emergency Preparedness

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CONTENTS

INTRODUCTION

HCN and simple cyanide (NaCN, KCN) are well known as very toxic compounds, highly lethal for most living organisms. The toxic effect of cyanide on the body is due to its high affinity to metals and especially due to the inhibition of the enzyme cytochrome c oxidase, which leads to the disruption of cellular respiration and, in consequence, to tissue hypoxia and acidosis, and to the disturbance of the nervous system.

Currently, there are a number of industries associated with the use of cyanide or the formation of cyanide as a by-product. Accordingly, cyanide and other hazardous substances are present in the effluents of these industries. Cyanides are used extensively in the mining of precious metals and in the manufacture of pharmaceuticals, chemicals, pesticides, and plastics. In the gold/silver mining and jewelry industries, cyanides are used for the selective extraction of the precious metals from ores and for electroplating processes,

respectively (Luque-Almagro et al., 2016). Wastewater from agriculture may also contain cyanides originated by the decomposition of plant cyanoglycosides and cyanolipids during the processing of some raw materials (cyanide-containing crops, such as cassava, millet, etc.). These factors, among others, cause cyanide contamination of soil, groundwater, and other environments, which is hazardous for population.

The methods currently used to treat cyanide-containing waste (physicochemical, chemical) have disadvantages such as a high consumption of chemicals or energy and the generation of secondary waste. Bioremediation is an environmentally friendly alternative or complement to abiotic methods, and the search for new green methods and options for an efficient bioremediation of cyanide waste is needed (Anning et al., 2020). This work focuses on the search for enzymes that participate in the detoxification of HCN, on their function and their potential uses.

The enzymes of interest largely belong to the nitrilase superfamily. This group includes 13 branches, and its known members catalyze the hydrolysis of non-peptide carbon-nitrogen bonds. The branches differ in the amino acid sequence and the catalytic activity. The most studied and best characterized enzymes of the nitrilase superfamily are, e.g., nitrilase (NLase; EC 3.5.5.1), amino-terminal amidase (EC 3.5.1.-), aliphatic amidase (EC 3.5.1.4), biotinidase (EC 3.5.1.12), β-ureidopropionase (3.5.1.6), and D- and L-carbamoylase (EC 3.5.1.77; EC 3.5.1.87). Members of this superfamily are involved in the biosynthesis and modification of various natural compounds and are found in bacteria, fungi, plants, and animals (Pace and Brenner, 2001).

This work focuses on the enzymes of the first branch, the so-called "true NLases" (hereafter referred to as NLases), in particular their subtypes involved in the detoxification of HCN. Different subtypes of NLases are specific for certain bacteria, fungi, or plants.

The functions of NLases in nature seem to mainly involve plant-microbe interactions. For example, microbial NLases may participate in the synthesis of some plant hormones or detoxify and metabolize plant nitriles. At the same time, microbial NLases may take part in some of the mechanisms that promote microbial parasitism in plants containing nitrile compounds (Howden et al., 2009; O'Reilly and Turner, 2003).

NLases catalyze nitrile hydrolysis and have a wide range of substrate specificities, so they are able to process nitriles of different structures as substrates: aliphatic nitriles (e.g., β-cyano-L-alanine (AlaCN), acrylonitrile), aromatic nitriles (e.g., cyanopyridine, benzonitrile), and arylaliphatic nitriles (e.g., 3-phenylpropionitrile) (Figure 1) (Brenner, 2002; O'Reilly and Turner, 2003). Cyanide hydratase (CynH) and cyanide dihydratase (CynD) catalyze the cyanide transformation into amide and acid products (formamide and formic acid), respectively (O'Reilly and Turner, 2003).

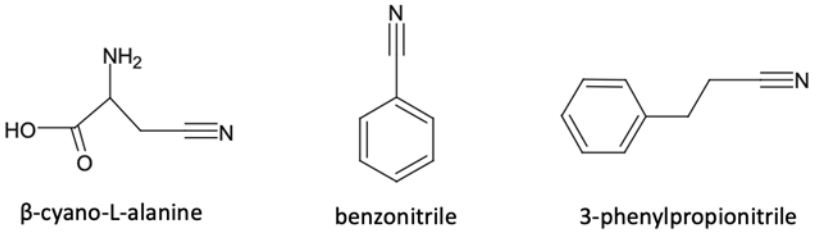

Figure 1. Examples of structurally different nitriles

NLases have a wide range of potential applications in various fields. One reason for this is their environmental compatibility. The biotechnological importance of NLases is very high in the production of chemicals and in the degradation of waste materials. Biohydrolysis of nitriles and bioremediation of cyanides by NLases are promising routes due to the high catalytic activities and suitable substrate specificities of the enzymes. Since the discovery of microorganisms possessing nitrile-hydrolyzing enzymes, many papers have been published on the biochemical and genetic background of the nitrile transformation process, the enzyme structures, and the metabolic

pathways, in which the enzymes are involved (Sharma et al., 2019; Kumar et al., 2017).

In this thesis we discuss the potential of these enzymes in the context of the current state of cyanide remediation and up-to-date trends in alternative solutions. The source of the enzymes in this work was Basidiomycota fungi, a group of organisms known for their broad biodegradation potential. However, the potential of this group of fungi for the detoxification of cyanide has been poorly explored so far. Basidiomycota were found to be a rich source of plant NLase homologues (NIT4) involved in the hydrolysis of AlaCN, an intermediate of HCN scavenging. In addition, Basidiomycota were found to produce some CynHs, which directly convert HCN. The biochemical and catalytic properties of the enzymes were studied after selected representatives of these enzymes were obtained by heterologous expression of synthetic genes (Rucká et al., 2020; Sedova et al., 2021).

In addition, the CynHs were used as purified enzymes to study their ability to work in media contaminated with cyanide. Future uses of the enzymes for environmental technologies and bioanalytics were discussed, comparing their properties with alternative chemical and biological tools.

AIMS OF THE THESIS

The primary aim of this thesis was the preparation of new biocatalysts for the biodegradation of free cyanide and the evaluation of their suitability for future uses such as the remediation of industrial wastewater or of contaminated environment, for the sake of population protection.

Specific aims were:

- investigate the cyanide detoxification potential of fungi focusing on Basidiomycota;
- express selected genes focusing on cyanide hydratase and nitrilase NIT4;
- determine the catalytic properties of the enzymes;
- investigate the enzyme operation in cyanide-contaminated media.

METHODS

Genes, their expression and protein purification

The sequences of the genes encoding for putative NLases and CynHs were found in GenBank and optimized in terms of the codon preference of *Escherichia coli*. The genes were synthetically prepared (GeneArt, ThermoFisher Scientific) and overexpressed in *E. coli*. The proteins were purified and examined for their activities as described below.

The genes encoding for NitAb (GenBank: XP_006462086.1), NitTv1 (GenBank: XP_008032838.1), NitSh (GenBank: XP_007307917.1), and NitEg (GenBank: KZV92691.1) enzymes were ligated into vector pET22b(+), which was then used to transform *E. coli* Origami B (DE3) competent cells as previously described for NitSh (Rucká et al., 2019). The gene overexpression was induced with IPTG, and the proteins were purified by cobalt affinity chromatography on

TALON® Metal Affinity Resin (Clontech), as described for other nitrilases (Rucká et al., 2020).

Enzyme Assays

Activity assays were carried out in 1.5 ml Eppendorf tubes at 30 °C with or without shaking (850 rpm; Eppendorf ThermoMixer Comfort (Eppendorf).

Specific enzyme activities were determined with AlaCN as substrates for NLases and with KCN or with 2-cyanopyridine (2CP) as substrates for CynHs. Kinetic parameters V_{max} and K_M were calculated using the MyCurveFit program (https://mycurvefit.com). To determine the temperature and pH stability of the enzymes, as well as their pH and temperature optima, cinnamonitrile (CiN) and 2-cyanopyridine (2CP) were used as substrates for NIT4 and CynHs, respectively (see Table 1 and Table 2 for details.

Table 1. NIT4 nitrilase assays – method details

	Substrate (mM)	Reaction conditions	Compound determined	Analysis
Standard assay	AlaCN (25)	pH 8.0 [b], 30 °C, 5-10 min	Ammonia	Photometry
Kinetics	AlaCN (2.5–25)			
Relative activity	Aryl(aliphatic) nitriles (25) [a]		Acid, amide	HPLC
Temperature optimum	CiN (25)	pH 8.0 [b], 20–50 °C, 5-10 min	Acid, amide	HPLC
Temperature stability		Pre-incubation: pH 8.0 [b], 20-50°C, 2 h; Reaction pH 8.0 [b], 30 °C, 10 min		
pH optimum		pH 4.0-11.2 [c], 30 °C, 10 min		
pH stability		Pre-incubation: pH 4.0-11.2 [c]; 2 h, 30 °C; Reaction: pH 8.0 [c], 30 °C, 10 min		

Abbreviations: AlaCN – β-cyano-L-alanine, CiN – cinnamonitrile

[a] Allylcyanide, fumaronitril, indole-3-acetonitrile, phenylacetonitrile, 4-phenylbutyronitrile, 3-phenylpropionitrile, and phenylthioacetonitrile.

[b] 50 mM Tris/HCl buffer, pH 8.0, 150 mM NaCl

[c] Britton–Robinson buffers

Table 2. Cyanide hydratase assays - method details

	Substrate (mM)	Reaction conditions	Compound determined	Analysis
Standard assay	KCN (25)	pH 9.0 [a], 30 °C, 1-2 min	Residual cyanide, formamide	Photometry
Kinetics	KCN (2.5–25)			
Thermostability (long-term)	KCN (25)	Pre-incubation: 1-24 h at 27, 37, 43, 50 °C; Reaction: standard assay		
Cyanide conversion	KCN (0.6, 4.6, 25, 100)	pH 9.0-10.5, 30 °C, 5–180 min		
Temperature optimum	2-Cyanopyridine (25)	pH 8.0 [b], 20-50 °C, 10 min	Picolinic acid, picolinamide	HPLC
Thermostability (short-term)		Pre-incubation: 20-50 °C, 2 h Reaction: pH 8.0 [b], 30 °C, 10 min		
pH optimum		pH 4.0–10.8. 30 °C, 10 min		
pH stability		Pre-incubation: pH 4.0–10.8, 2h, 30 °C, Reaction: pH 8.0 [b], 30 °C, 10 min		

[a] 100 mM glycine/NaOH, pH 9.0-10.5

[b] 50 mM Tris/HCl buffer, pH 8.0, 150 mM NaCl

The degree of free cyanide (fCN) conversion was studied under the following conditions: 100 mM glycine/NaOH pH 9.0, 9.5, 10.0, or 10.5. The simulated wastewater was prepared according to the literature (Jarrah and Mu'azu, 2016; Papadimitriou et al., 2009).

The following concentrations of fCN were used: 0.6, 4.6, 25 or 100 mM (added as KCN). Optionally, model compounds were added to the reaction mixtures - Na_2S, KSCN, NH_4Cl, phenol, $CuSO_4$, or $AgNO_3$ (see Table 3 for details). Reactions were also performed in the same buffer with 0.6 mM fCN or 4.6 mM fCN without other additives. Enzyme concentrations were 2.5 μg/ml and 5.0 μg/ml for the reactions in model coke plant and petrochemical effluents, respectively. Controls did not contain the enzyme. Using the picric acid method (see below) or the Spectroquant® kit (Supelco), the residual fCN was determined.

Table 3**.** Composition of simulated wastewaters

Wastewaters	Pollutant, mM						
	free CN	S^{2-}	SCN^-	NH_4^+	Phenol	$AgNO_3$	$CuSO_4$
Coke oven	0.6	0	8.62	10.7	12.8		
Petrochemical	4.6	23.4	4.61	2,5	0.64		
Electroplating	100					1	1

Analytical Methods

The concentrations of nitriles and their reaction products were determined using HPLC:

- column ACE C8 (5 µm, 250 mm × 4 mm; Advanced Chromatography Technologies Ltd.); mobile phase containing 10% acetonitrile in 5 mM sodium phosphate buffer, pH 7.2; flow rate 0.9 ml/min
- column Chromolith SpeedRod RP-18 (50 mm × 4.6 mm; Merck KgaA, Darmstadt, Germany); mobile phase was containing 20% acetonitrile and 0.1% phosphoric acid in water; flow rate 2 ml/min.

Spectrophotometric measurements were used to determine fCN or formamide by the picric acid method (Fisher, 1952) and by the hydroxylamine method (Cluness, et al. 1993), respectively. Absorbance was measured at 520 nm and 540 nm, respectively.

SDS-PAGE electrophoresis was performed according to Laemmli (1970), using a 10% polyacrylamide gel and standards with molecular weights of 14.4–97 kDa (GE Healthcare). The gel was stained with brilliant blue P-250.

RESULTS

Nitrilases NIT4

Basidiomycota genomes contain a large number of genes encoding for potential proteins of the “nitrilase superfamily” (Rucká et al., 2019). The enzymes NitTv1 (sequence XP_008032838.1) and NitAb (sequence XP_006462086.1) from *Trametes versicolor* and *Agaricus bisporus*, respectively, were selected as the representatives of the fungal NIT4 NLase homologues to study their functions.

Both enzymes were investigated for their catalytic properties (Table 4). The specific activities for 25 mM substrate (AlaCN, PPN) and kinetic parameters for AlaCN were calculated based on ammonia production, which was determined spectrophotometrically. The ratio of activities for AlaCN and PPN were also determined, which is characteristic of each NIT4 enzyme.

Table 4. Catalytic properties of fungal nitrilases NitTv1 and NitAb

Enzyme	Substrate AlaCN					AlaCN:PPN Activity
	specific activity [U mg^{-1} protein]		V_{max} [U mg^{-1} protein] K_M (mM)		NHase:NLase Activity	
	Asparaginase + (Total Activity)	Asparaginase − (NLase Activity)	NLase Activity	NHase Activity		
NitTv1	131.5 ± 0.5	94.2 ± 1.0	129.8 ± 11.4 7.72 ± 1.82	53.2 ± 9.2 6.74 ± 3.35	0.40 ± 0.02	91 ± 3
NitAb	40.1 ± 0.1	26.8 ± 0.4	34.8 ± 2.0 7.38 ± 1.40	19.6 ± 2.5 4.97 ± 2.12	0.50 ± 0.02	56 ± 4.0

Abbreviations: AlaCN - β-cyano-L-alanine, PPN – 3-phenylpropionitrile, NHase – nitrile hydratase, NLase – nitrilase.
Note: Determined with 25 mM substrates at pH 8 and 30 °C.

NIT4 NLases have dual catalytic activity: NLase (the reaction products are the corresponding carboxylic acid and ammonia) and nitrile hydratase (NHase; the reaction product is the corresponding amide). Thus, dual-activity NLases produce an acid and an amide as reaction products. Accordingly, NIT4 converts AlaCN to Asp and ammonia or to Asn (Figure 2).

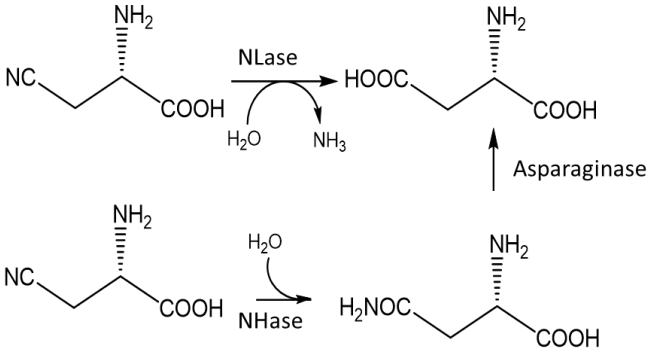

Figure 2. Conversion of β-cyano-L-alanine by NIT4 nitrilase to Asp and ammonia or to Asn. Asparaginase converts Asn to Asp.

Aliphatic and arylaliphatic nitriles were used as substrates for the studied enzymes (Table 5). The formation of reaction products - carboxylic acids and amides - was determined using HPLC. Relative enzyme activities with the above substrates were calculated using AlaCN as the reference substrate.

Table 5. Comparison of substrate specificities of NitTv1 and NitAb nitrilases

Enzyme	Relative activity [%]
NitTv1	**β-cyanoalanine (100),** fumaronitrile (8.6), 4-cyanopyridine (1.7), cinnamonitrile (1.6), 3-phenylpropionitrile (1.1), phenylacetonitrile (1.0)
NitAb	**β-cyanoalanine (100)**, cinnamonitrile (3.3), 3-phenylpropionitrile (1.8), phenylacetonitrile (1.1), fumaronitrile (<1), 4-cyanopyridine (<1)

Note: Determined with 25 mM substrates at pH 8 and 30 °C. Specific activities of 131.5 and 40.1 U/mg of protein for β-cyanoalanine were taken as 100% in NitTv1 and NitAb, respectively.

Studies of the influence of temperature and pH on the activity and stability of the enzymes were carried out using CiN as substrate. For NitTv1, the NHase: NLase ratio was affected by the reaction temperature, i.e., an increase in temperature led to an increase in this ratio. A pH shift to acidic also led to an increase in the NHase:NLase ratio. The effect of pH on the enzyme activity and stability did not differ significantly between NLase and NHase activities for NitAb enzyme, in contrast to more significant differences in the NitTv1 enzyme.

Cyanide hydratases

The specific activity calculated from the amount of formamide produced was 784 U/mg protein and 206 U/mg protein for the purified NitEg and NitSh enzymes, respectively. Formamide is the main product of the CynH-catalyzed reaction of HCN (one form of fCN formed by dissolution of simple cyanide) (Figure 3).

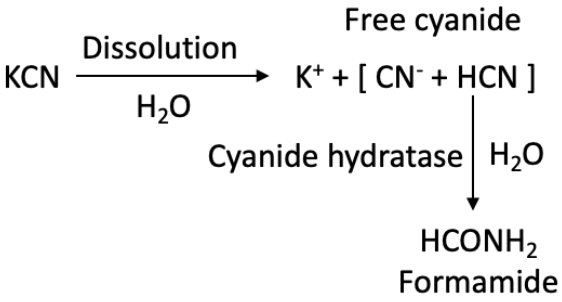

Figure 3. Free cyanide detoxification catalyzed by cyanide hydratases.

NitEg is a more promising enzyme for biotechnology in terms of its biochemical parameters. Comparing the effect of temperature

and pH on the activity of the two enzymes, NitEg had a wider range of activity and stability. For the NitEg enzyme, the influence of other factors (accompanying pollutants) on its activity was also determined, as these can affect the real working conditions of the enzyme, when it is used for the biodegradation of cyanide in an industrial wastewater.

The presence of copper ions (0.1 mM) in the reaction mixture did not significantly reduce the enzyme activity, but the activity of the enzyme was significantly inhibited at concentrations of copper ions higher than 5 mM. Silver ions (0.1 mM) already exhibited an inhibitory effect on the enzyme. Nevertheless, the conversion of fCN in the presence of 1 mM Cu^{2+} or 1 mM Ag^{+} was feasible (see below).

The specific activity of the purified enzyme was maintained for 98 days when the enzyme was stored at 4 °C. A slight but statistically significant decrease was observed after day 69, while after 98 days the enzyme activity was still approximately 83% of the original value (Figure 4).

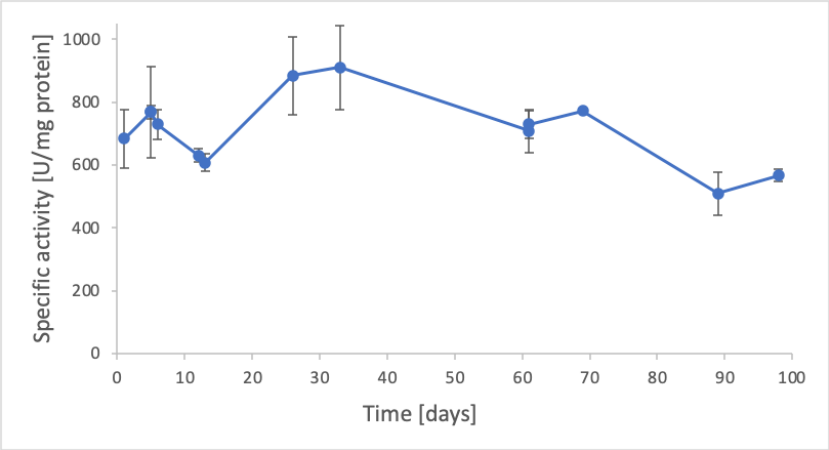

Figure 4. Stability of NitEg during storage at 4 °C. Specific activity was determined by picric acid method.

Investigations of enzyme activity were carried out at pH from 9.0 to 10.5 using 4 and 20 µg enzyme per ml. The efficiency of NitEg in a concentration of 4 µg of enzyme per ml and at pH 9.0 to eliminate 25 mM fCN was high - after 1 hour the cyanide was completely removed. With an increase in pH, the efficiency of the enzyme decreased: at pH 9.5, 89% of cyanide was removed within one hour, but only 54% at pH 10.0. At pH 10.5, the enzyme had a very low efficiency.

The performance of an increased concentration of NitEg (20 μg per ml) was investigated at the same pH values (9.0-10.5). At pH 9.0 and 9.5, fCN was almost completely removed after only 10 and 30 min, respectively. At pH 10, about 80% of cyanide was removed within one hour. Even at pH 10.5, some decrease in the amount of cyanide (about 20%) was observed.

According to literature data (Papadimitriou et al., 2009), we prepared a mixture simulating a wastewater from coke production with certain concentrations of phenol, CN^-, SCN^-, and NH_4^+; the fCN concentration was 0.6 mM, and pH was 9.1. The enzyme (2.5 μg/ml) decomposed fCN almost completely in 90 min.

Another model effluent was prepared to simulate the composition of wastewater from petrochemical industry (Jarrah et al., 2016). This mixture contained a significant concentration of S^{2-}, while the concentration of fCN was 8-times higher than in the above coke-plant effluent model. Both model effluents contained phenol and

ammonia, but the petrochemical effluent model contained them in lower concentrations, and it did not contain SCN^-. Under conditions simulating petrochemical wastewater, 96% fCN was removed after 45 min by the enzyme at 5 μg/ml.

To study the function of the enzyme under the conditions of high fCN concentration, we studied the biocatalytic degradation of a 100 mM fCN solution (simulating a diluted effluent) using NitEg at various concentrations (14, 20, and 30 μg/ml) at pH 9. The obtained results demonstrate that even 14 μg of the enzyme per ml is sufficient to degrade more than 97% of cyanide within 60 min. However, approximately 2% of fCN was not removed during the entire experiment time of 3 hours, even at an enzyme concentration of 30 μg per ml. According to the data obtained in the control sample, the loss of cyanide associated with abiotic factors was about 17%.

The enzyme activity in simulated electroplating effluents was studied at a concentration of fCN of 100 mM and a concentration of

copper and silver of 1 mM. The percentage of removal of fCN in the presence of metal ions was 96-98% and was similar to that in the reaction without metals. In the presence of metals, the initial reaction rate was slightly lower.

CONCLUSIONS

The aim of the thesis was to evaluate the current status of cyanide wastewater treatment and to assess the importance of bioremediation enzymes (NLases, CynHs) for the detoxification of hazardous cyanide. The literature survey shows that the trend in the cyanide effluent remediation is towards processes combining various principles (physical, chemical, biological). However, the influence of enzymatic processes on this area of environmental technology is still limited and rather marginal. To contribute to the development of the enzymatic processes, which are friendly to the environment and population, in this thesis we have identified new suitable bioremediation enzymes *in silico*, we produced them as recombinant proteins and evaluated their catalytic properties.

The first part of the study focuses on the search for homologues of plant NLases in fungi and their characterization including the identification of their substrate-specificity subtype. NLases NitTv1 and NitAb, homologues of NIT4 according to sequence,

were confirmed to act on β-cyano-L-alanine (AlaCN), the key intermediate of fCN metabolism in plants. The fungal enzymes exhibited a broader substrate specificity compared to plant NIT4, combining features of the plant NIT1–NIT3 and NIT4 types. Thus, these NLases can increase the resistance of fungi to other natural (plant) nitriles, which are generally toxic. The new fungal NLases produce amides and/or carboxylic acids from these substrates, with one of them, the NitTv1 enzyme, showing activity and stability over a wide pH range.

The second part of the study is dedicated to two fungal CynHs, analyzing their operating conditions, optimum and stability to identify the most promising candidate for cyanide waste processing. The NitSh and NitEg enzymes are the first CynHs described in Basidiomycota, with NitEg emerging as the superior candidate. This enzyme shows a high specific activity and is resistant to alkaline pH, tolerates high concentrations of fCN and other industrial pollutants, and is stable

during a long-term storage. Its potential application as an effective biocatalyst under alkaline conditions extends to the development of analytical tools – cyanide bioassays and biosensors. This work has created a basis for further research of the potential of bioremediation tools in the detoxification of wastewater and in analytics of hazardous chemicals such as cyanides, detrimental for human health.

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Own relevant publications

The dissertation is based on the following articles:

1. Rucká L, Kulik N, Novotný P, **Sedova A**, Petrásková L, Příhodová R, Křístková B, Halada P, Pátek M, Martínková L. Plant nitrilase homologues in fungi: phylogenetic and functional analysis with focus on nitrilases in *Trametes versicolor* and *Agaricus bisporus*. Molecules **2020**; 25(17): 3861. doi: 10.3390/molecules25173861. IF = 4.4

2. **Sedova A**, Rucká A, Bojarová P, Glozlová M, Novotný P, Křístková B, Pátek M, Martínková L. Application potential of cyanide hydratase from *Exidia glandulosa*: Free cyanide removal from simulated industrial effluents. Catalysts **2021**; 11(11): 1410. doi: 10.3390/catal11111410. IF = 4.5

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Proceedings and abstracts:

Sedova A., Rucká L., Glozlová M., Novotný P., Martínková L., Bojarová P. Enzymatic detoxification of cyanide using cyanide hydratases. 15th Annual Doctoral Conference Proceedings, New Approaches to State Security Assurance, 11 February 2021, online. Book of abstracts: page

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BioTech 2020 (June 16-19, 2021) B. Křístková, **A. Sedova**, P. Bojarová, M. Glozlová, L. Martínková. Exploring cyanide-degrading enzymes in fungi. BioTech 2020 and 8th Czech-Swiss Symposium, 16.6.-19.6.2021, Prague (CZ), online Book of abstracts: page 98, poster P32.

L. Martínková, **A. Sedova**, R. Rädisch, L. Rucká, N. Kulik, P. Bojarová, M. Pátek: Biotechnology potential of aldoxime- and nitrile-converting enzymes, BioTech 2020 and 8th Czech-Swiss Symposium, 16.6.-19.6.2021, Prague (CZ), online. Book of abstracts: page 70, oral presentation L51.

SUMMARY

Wastewater from certain industrial processes (gold and silver mining, electroplating with Cu, Ag and other metals, coal coking) contains high concentrations of free cyanide (hydrogen cyanide and cyanide ions; fCN) and can pose a threat to environmental safety and health of population. Modern approaches to wastewater treatment are primarily based on (physico)chemical methods and a final biological treatment with mixed cultures. Enzymatic removal of fCN represents an environmentally promising but underdeveloped approach.

This thesis focuses on the remediation potential of enzymes from the nitrilase superfamily. In fungi, hypothetical nitrilases and cyanide hydratases were found through database searches. Selected enzymes were overproduced in *Escherichia coli*, purified and characterized, and their remediation potential was evaluated.

Two of the enzymes belonged to the NIT4-type nitrilases (EC 3.5.5.4) according to their amino acid sequences: the NitTv1 enzyme from the fungus *Trametes versicolor* and the NitAb enzyme from thus fungus *Agaricus bisporus*. NIT4-type nitrilases are widespread plant enzymes that transform β-cyano-L-alanine (AlaCN), an intermediate of fCN scavenging. NIT4 detoxify AlaCN and simultaneously convert it to utilizable amino acids. In this work, these enzymes were described for the first time in fungi. The fungal enzymes NitTv1 and NitAb exhibited high specific activities for AlaCN. Moreover, the substrate specificity of these enzymes went beyond plant NIT4, as they could also transform other nitriles (3-phenylpropionitrile, cinnamonitrile, fumaronitrile). The stability was better for NitTv1 than NitAb. Presumably, these nitrilases play a role in plant-fungus interactions, allowing fungi to detoxify plant nitriles.

Cyanide hydratases (CynHs) (EC 4.2.1.66) convert fCN into the much less toxic formamide and have been therefore considered for the decontamination of cyanide effluents. The purified CynH from

Exidia glandulosa (NitEg) showed high activity towards fCN. Its pH and temperature optimum were 6–9 and 40–45 °C, respectively. Thus, the catalytic properties of NitEg surpassed those of the other CynH studied, which was from the fungus *Stereum hirsutum* (NitSh). Although silver and copper ions at 1 mM reduced the activity of NitEg by 30–40%, an almost full conversion of fCN was achieved in their presence. Phenol, thiocyanate, sulfide, and ammonia, at levels typical for industrial effluents, had no negative effect on fCN conversion either. The enzyme was functional at high fCN concentrations (100 mM), which is relevant to the remediation of wastewater from metal electroplating.

NitEg is thus a promising biocatalyst for direct fCN detoxification, and NIT4 was shown to likely play an important role in fCN detoxification by fungi. Both enzymes are also promising for the detection and determination of fCN. Nevertheless, the potential of the enzymes is just emerging and requires intensive studies to improve and scale up their production and application.