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Biotransformation of free cyanide to formic acid by a cyanide hydratase–formamidase cascade reaction

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ABSTRACT

Free cyanide (fCN) consisting of HCN and CN^- is highly hazardous. Today, removal of cyanide from industrial (mining, plating, coke-plant) wastewaters largely relies on physicochemical processes followed by microbial degradation. Enzymatic processes are gaining ground but are still at a low technological stage. The cyanide-converting enzymes of interest are primarily cyanide dihydratases (CynDs; EC 3.5.5.1.), which hydrolyze HCN to formic acid and ammonia, and cyanide hydratases (CynHs; EC 4.2.1.66), which hydrate HCN to formamide. CynHs usually have much higher specific activities and a broader pH profile especially in the alkaline region compared to CynDs. However, the product of CynH, formamide, although much less toxic than fCN, still poses a significant health risk. Therefore, it is attractive to combine the CynH with an amidase that converts formamide to formic acid and ammonia. Here we demonstrate on a laboratory scale a two-step "one-pot" detoxification of fCN (5 mM) to formic acid using recombinantly produced purified enzymes – CynH from *Exidia glandulosa* and formamidase (EC 3.5.1.49) from *Bacillus cereus*. The reaction proceeds at pH 9.0–10.0, which reduces the risk of HCN escape. We also hypothesize that the cascade can be used for fCN determination after coupling an NAD-dependent formate dehydrogenase.

1. Introduction

Free cyanide (fCN) occurs in two forms, HCN and CN^-, the ratio of which depends on the pH value [1]. The release of fCN into the environment is caused by industry, agriculture, combustion vehicle traffic, fires, or smoking. Simple cyanide (KCN, NaCN) is industrially used in, e.g., gold and silver mining, metal electroplating, jewelry manufacturing, and organic syntheses. In addition, fCN is formed during coking of coal and smelting of iron. Industrial wastewaters may contain fCN in concentrations ranging from mg/L (e.g., coke oven and iron smelting wastewaters) to several tens of g/L (e.g., plating and jewelry making wastewaters) [2]. Some cyanide wastes also contain metal cyanide complexes, which are usually less toxic but may decompose to fCN. Large quantities of cyanide are used in gold mining, and cyanide residues with up to ≈10 mg of total cyanide (sum of free and complexed cyanide) per L are found in the disposed waste ("tailings") [3]. The reason for the high toxicity of fCN is its complexation with metals in metalloproteins. Especially, binding to cytochrome c oxidase inhibits respiration [4], which is threatening.

The treatment of cyanide effluents has been reviewed in several studies (e.g., [1,2,4–6]). Oxidation of cyanide to cyanate or complexation and precipitation of cyanides are the main processes for the detoxification of fCN. Partially treated wastewaters can be further remediated using microbial consortia. The natural degradation of cyanide in gold mine tailings by indigenous bacteria is also a promising way to address the problem [3]. The tailings are a valuable source of strains

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suitable not only for the degradation of cyanide but also for the biotransformation of certain nitriles [7].

In contrast, the use of isolated cyanide-transforming enzymes in the treatment of cyanide wastewaters is still technologically immature although the abundance of various cyanide-converting enzymes is recognized as the basis for optimizing the biodetoxification of cyanide [8]. The use of enzymes instead of growing cells may be particularly advantageous for the degradation of toxic compounds [9].

The candidate enzymes are primarily cyanide dihydratase (EC 3.5.5.1; CynD) and cyanide hydratase (EC 4.2.1.66; CynH) [10–12]. Both are members of the nitrilase superfamily (branch 1) but differ in specific activities, pH profiles, or stabilities. CynHs exhibit specific activities that are much higher than those of CynDs (hundreds to thousands of U/mg in CynHs, tens of U/mg in CynDs). In addition, CynHs are active at a higher pH than CynDs. CynHs are typically active at pH values of up to about 10.5 [13,14]. Wild-type CynD from *Bacillus pumilus* is already almost inactive at pH 9.0. CynHs also outperform CynD mutants, some of which have an improved activity at pH ≈ 9–9.5 [15–17]. However, the product of CynHs, formamide, is problematic for health reasons, as it has been suspected of being toxic to development or reproduction in mammals [18].

A potential solution to this problem is the combination of CynH with an amidase that converts formamide into formic acid and ammonia, which are relatively harmless at low concentrations. This type of cascade has already been demonstrated for immobilized cells of *Fusarium oxysporum* and *Methylobacterium* sp. with CynH and formamidase (AmiF) activity, respectively, in a flow mode [19]. However, the apparent V_{max} values of both enzymes – ≈20 μmol/min/g of cell dry weight [19] – were modest.

In this study, we aimed to demonstrate the two-step conversion of fCN into benign compounds using purified enzymes. We hypothesized that replacing whole cells with purified CynH and AmiF could significantly improve the efficiency of the process. Several CynHs were overproduced in *Escherichia coli* and exhibited excellent specific activities [13,14,20]. For example, we recently reported a new CynH from *Exidia glandulosa* (NitEg enzyme) with a high activity and stability and a broad pH range [14]. Thus, this enzyme was chosen to catalyze the first step of the cascade. Experimentally confirmed formamidases (AmiF; EC 3.5.1.49) suitable for the second step were few despite the large number of hypothetical AmiFs. However, an AmiF from *Bacillus cereus* [21] (enzyme BceAmiF) was promising, as its specific activities and pH profile were comparable to those of NitEg. We investigated the compatibility of the two enzymes and the possibility of combining them in a one-pot reaction on a laboratory scale.

2. Materials and methods

2.1. Enzymes and chemicals

Talon® Metal Affinity Resin was purchased from Clontech Laboratories, Inc. Formic Acid Assay Kit was purchased from Megazyme. All chemicals were of the highest purity available and were obtained from standard suppliers.

2.2. Sequence searches and phylogenetic tree construction

Homologs of AmiF were searched for using BLAST [22], BRENDA [23], and InterPro [24]. The complete dataset was used for tree construction with MEGA X software [25]. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [26]. Positions with less than 90% site coverage were eliminated (327 positions were kept in the final dataset). iTOL on-line service [27] was used for visualization and storage of data.

2.3. Gene expression and enzyme purification

Cyanide hydratase NitEg (GenBank: KZV92691.1) and formamidase BceAmiF (pdb code: 5G3O_A) were produced as fusion proteins containing His_6-tag at their C-termini [14,21].

The optimized gene encoding NitEg was expressed in *E. coli* and the enzyme was purified as described previously [14]. Purified NitEg was stored in 50 mM Tris/HCl buffer, pH 8.0, with 150 mM NaCl, at 4 °C, for ≤ 2 months (no significant loss of activity was found within this period [14]).

The sequence of the *amiF* gene was optimized by GeneArt, Thermofisher (Supplementary Information, Fig. S1). The expression strain and expression vector were *E. coli* BL21(DE3) and pET22b(+), respectively, as in the previous work [21]. The cultivation conditions [21] were modified: the culture was grown in 200 mL of 2xYT medium. Initially, the cultivation temperature was 37 °C. Optical density (OD) was monitored at 600 nm. After OD reached 1.0, 0.02 mM IPTG was added, and cultivation temperature was lowered to 16 °C; cultivation was performed for a further 20 h. The enzyme was purified from the cell-free extract (CFE) by immobilized metal affinity chromatography (IMAC) as described previously [21]. The preparation of CFE by cell sonication was modified in sonic power and buffer composition. Cells were harvested by centrifugation and washed with 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (buffer P). The cells were resuspended in 10 mL of buffer P and sonicated (Bandelin Sonopuls HD2200; 20% of maximum power, 4 °C, 6 × 30 s). After centrifugation (27,000 g, 2 × 15 min, 4 °C), the volume of CFE was adjusted to 20 mL with buffer P. CFE was divided into two 10-mL portions; each portion was mixed with 2 mL of Talon resin and incubated for 2 h at 4 °C. The resin was then transferred to an empty column and washed with 2 mM Tris/HCl, pH 8.0, with 100 mM NaCl (*i*) without imidazole, (*ii*) with 10 mM imidazole, and (*iii*) with 50 mM imidazole. Active fractions eluted with 50 mM imidazole were pooled, and concentrated on Amicon 30 K (4000 g, 4 × 10 min, 4 °C), while the elution buffer was exchanged for 100 mM citrate buffer, pH 6.0 (buffer C). Analogously, the enzyme was purified on a 5-mL His-Trap column (GE Healthcare). Purified BceAmiF was stored in buffer C on ice.

2.4. Determination of enzyme activities

The reactions proceeded in 1.5-mL minitubes (with 0.5-mL working volume) incubated in Thermomixer Eppendorf Compact.

The activity of NitEg was determined as described previously [14] but the reaction was stopped by adding 1 mL of 0.2 M NaOH (instead of methanol) per 0.5 mL of the reaction mixture. Residual fCN was determined by picric acid method as described previously [14]. One unit of NitEg activity was defined as the amount of enzyme that converted 1 μmol of fCN per 1 min at pH 9.0 and 30 °C.

The activity assay of BceAmiF was based on a previous protocol [21] modified in terms of buffer and reaction temperature. Buffers were Britton-Robinson (buffer B-R), pH 8.0−10.0, consisting of acetic acid, boric acid, phosphoric acid (40 mM each) and 0.2 M NaOH mixed in suitable proportions. The purified enzyme (0.2–2.2 μg) was pre-incubated in buffer at 30 °C for 10 min. The reaction was started with 100 mM formamide (final concentration) and was stopped after 15 min with a ninefold volume of 1% H_3PO_4. Formamide was determined photometrically [14]. One unit of AmiF activity was defined as the amount of enzyme that converted 1 μmol of formamide per 1 min at pH 9.0 and 30 °C.

2.5. Enzymatic degradation of cyanide

Reactions catalyzed by CynH were performed in 2-mL minitubes placed in Thermomixer Eppendorf Compact (350 rpm), at 30 °C. Reaction mixture (1 mL) contained 100 mM glycine/NaOH (buffer G), pH 9.0, 9.5 or 10.0, 5 mM KCN, and purified NitEg (5.8 μg/mL for pH 9.0,

9.5 or 10.0, and 18 μg/mL for pH 10.0). Reactions were started after 5-min preincubation by adding KCN from a stock solution of 500 mM KCN in buffer G, pH 9.0. The stock solution was freshly prepared each day. The reaction was stopped by adding 0.2 mL of 0.2 M NaOH per 0.1 mL of sample. fCN was monitored by picric acid method as described previously [14].

Reactions catalyzed by BceAmiF were performed in 2-mL minitubes placed in Thermomixer Eppendorf Compact (350 rpm), at 30 °C. The reaction mixture (0.5 mL) contained buffer G, pH 9.0, 9.5 or 10.0, 5 mM formamide and purified BceAmiF (7.3 or 14.6 μg/mL). Samples were withdrawn and the reaction was stopped as described above (Section 2.4). Formamide was determined photometrically as described previously [14]. The stock solution of formamide in water was freshly prepared each day.

"One-pot" two-step reactions were performed in 2-mL minitubes placed in Thermomixer Eppendorf Compact (350 rpm), at 30 °C. The reaction mixture (2 mL) contained buffer G, pH 9.0, 9.5 or 10.0, 5 mM KCN, purified NitEg (38.5 μg/mL) and purified BceAmiF (19.5 μg/mL). Reactions were started after 5-min preincubation by adding KCN to a final concentration of 5 mM. Samples were withdrawn and the reaction was stopped as described above. fCN and formamide were monitored as described previously [14]. Formic acid was determined photometrically using a Formic Acid Assay Kit. Absorbance at 340 nm (A_{340}) was monitored using a spectrophotometer UVmini-1240 (Shimadzu). NADH concentration was determined from the corresponding calibration curve (Supplementary Information, Fig. S2).

3. Results and discussion

3.1. Cyanide hydratase and amidase sequence space

3.1.1. Cyanide hydratase

Nitrilases are generally of prokaryotic origin, and the occurrence of *nit* genes in eukaryotes is due to horizontal transfer [8]. This was obviously followed by their diversification to give CynHs and other fungal nitrilases. According to searches performed about a year ago, CynHs form a group of almost 400 fungal enzymes, which share high levels of identity (largely >60%) [28].

The CynH catalyst used in the previous cascade was based on *Fusarium oxysporum* CCMI 876, a strain isolated from an industrial wastewater. The catalyst was comprised of whole cells, which were immobilized in alginate [19]. For the time being, the sequence of this CynH is unknown. To predict this sequence, with some probability, we searched for hypothetical CynHs in this species.

According to GenBank, nine hypothetical CynHs (RKK26290.1 and its homologs with 98% identities) are present in *F. oxysporum* strains. So far, the only characterized *F. oxysporum* CynH has been from strain N-10. However, its amino acid sequence was not reported except for its *N*-terminus (AITKFKAAAVTSEPGWFDLEGGVRKTIDFI) [29], which has been found to be highly conserved in eight of the nine hypothetical CynHs in *F. oxysporum*. A single amino acid residue, Y on the fifth position in the hypothetical CynHs, is replaced by F in strain N-10. The specific activity of the purified enzyme from strain N-10 was 0.36 U/mg protein for $K_2[Ni(CN)_4]$ and about 1000 times higher for KCN [29]. Another CynH (P32963.2 with 93% identity to RKL02403.1) was purified from *Fusarium lateritium*, with a specific activity of about 1109 U/mg protein for NaCN [30].

The CynH from *F. oxysporum* CCMI 876 was not extracted or purified. Therefore, we can only compare the whole-cell activity of this strain with an apparent V_{max} of 20 U/g dry cell weight [19] with *F. lateritium* with a specific activity of 19.6–102.5 U/mg dry cell weight. The reason for the different activities is not entirely clear. However, we can speculate on the effects of the induction conditions, which were different for each of the strains. *F. lateritium* was cultured in flow mode, and the highest activity was obtained when NaCN was fed at 6.0–6.4 mM/h for 36 hours. Under these conditions, the proportion of CynH relative to total cell protein was about 25%, whereas no activity was found in the absence of cyanide [30]. In contrast, *F. oxysporum* CCMI 876 was cultured for 6 days in batch mode without cyanide, and induction with 1 mM cyanide was initiated 16 hours before harvesting the biomass [19].

NitEg (KZV92691.1), a CynH we previously produced [14], was used in this work due to its advantageous properties. The purified enzyme was highly active with over 784 ± 32 U/mg determined at pH 9.0 and functional up to a pH of at least 10. The enzyme exhibited a remarkable storage stability with more than 80% of its activity retained after 98 days at 4 °C. Furthermore, NitEg was previously found to be functional in model industrial effluents containing sulfide, ammonia, thiocyanate and phenol, and in the presence of 1 mM Ag^+ or 1 mM Cu^{2+} [14]. It shows a significant sequence identity (86% identity, 95% coverage) with the CynH from *Neurospora crassa* (XP_960160.2), which is another CynH with significant activities in the alkaline region [13].

3.1.2. Formamidase

With a promising CynH in hand, we searched for a compatible amidase to hydrolyze the CynH product formamide to formic acid. In a previous report, amidases were classified into three clades [21]. Formamidases form two distinct clades – (*i*) AmiF-type enzymes belong to the nitrilase superfamily, whereas (*ii*) FmdS-type enzymes belong to the FmdA-AmdA family. The third amidase clade contains aliphatic amidases, acylamide amidohydrolases and other amidases of the nitrilase superfamily. Previously, the sequences were found to be 61 in total, out of them 21 in the AmiF clade [21].

The current number of putative AmiF-encoding sequences deposited in databases is much higher than reported in the study of 2011 [21]. We identified 653 putative AmiF sequences in our search after filtering out similar sequences with identities higher than 98% and sequences that lack the catalytic triad E-K-C (mandatory in the nitrilase superfamily). All remaining sequences also contained the second E recognized as a further catalytic residue [28]. Most of these sequences had a "CHDG" motif containing C as the catalytic residue. The H residue in the motif has been speculated to be an important feature of amidases [31]. The highest number of AmiF sequences was found in strains belonging to the orders Rhodobacterales/Hyphomicrobiales (over 230), Burkholderiales (over 108), and Cyanophyceae (over 103), followed by Bacillales (over 38) and eukaryotes – mainly fungi (38).

The database search was also helpful to predict the probable sequence of the previously used AmiF, whose sequence has not been reported. The catalyst was *Methylobacterium* sp. RXM CCMI 908, which was isolated from a sewage treatment plant [19]. Here we have found tens of putative AmiF sequences in various species of the genus *Methylobacterium*; they are members of the Rhodobacterales/Hyphomicrobiales clade (collapsed in the phylogenetic tree (Supplementary Information, Fig. S3, for online version see [32]). The formamidases of this genus show ≈ 48–50% identities to BceAmiF.

Two AmiF enzymes were previously characterized: BceAmiF from *Bacillus cereus* [21,33] and HpyAmiF from *Helicobacter pylori* [34,35]. They showed high specific activities of up to ≈2800 U/mg (at pH 6.0 and 50 °C) and ≈1040 U/mg (at pH 7.4 and 30 °C), respectively. Both were most active at pH 6.0 but retained some activity at pH 9.0 [21,35]. Members of the FmdS group such as FmdS from *Methylophilus methylotrophus* and *Paracoccidioides brasiliensis* exhibit much lower activities, i.e., 37.4 U/mg [36] and 2.73 U/mg [37], respectively.

BceAmiF with the highest specific activity reported was chosen for the further study. The enzyme was purified by IMAC with an excellent purity (Supplementary Information, Fig. S4). Furthermore, BceAmiF retained about 50% of its activity after 101 days (Fig. S5).

The optima of BceAmiF were pH 6.0 and 50 °C – the activity of the enzyme under these conditions was 2800 ± 500 U/mg protein [21]. However, the use of BceAmiF in the cascade required the enzyme to be active at an alkaline pH and lower temperature compatible with the handling of fCN. Therefore, we tested the activity of the enzyme at pH

8.0–10.0 and 30 °C (Fig. 1). The activities at pH 9.0 and 10.0 (in B-R buffers) were still very good at ≈790 U/mg and ≈400 U/mg, respectively. In addition, the enzyme retained approximately 98%, 85% and 71% of its activity in the presence of 1 mM, 5 mM and 10 mM KCN, respectively (Fig. 2). The pH profile and resistance to fCN indicated that the cascade could be performed in one pot. The activity of BceAmiF was not significantly affected by various metal cations (Mg^{2+}, Ca^{2+}, K^{+}, Na^{+}, Pb^{2+}) except for Hg^{2+} (2 mM each) [21]. However, the resistance of the enzyme to typical co-pollutants in fCN-containing effluents remains to be investigated.

BceAmiF was able to completely degrade 5 mM formamide, which is the maximum concentration of formamide that can occur during the cascade conversion of 5 mM fCN. The enzyme load was 7.3 μg (Figs. 3A) or 14.6 μg (Fig. 3B) per mL. The reaction catalyzed by the higher amount of enzyme was faster especially within the first 10 min at all pH values. At pH 9.0 or 9.5, the final conversions (95% to over 99%) were similar for both enzyme loads. At pH 10.0, the higher amount of enzyme provided a higher conversion (about 93%) than the lower (84%).

3.2. Single-step and two-step degradation of free cyanide

The conversion of fCN was previously catalyzed by NitEg. Reactions of 25 mM fCN were carried out at pH 9.0, 9.5 or 10.0 and 30 °C using 20 μg of purified enzyme per mL of the reaction mixture. The conversions were 100% at pH 9.0 and 9.5 after 30 min, and more than 80% at pH 10.0 after 1 hour [14]. Here, we investigated NitEg-catalyzed reactions of 5 mM fCN at pH 9.0, 9.5 or 10.0. An enzyme load of 5.0 μg/mL almost fully converted the substrate after 20 min at pH 9.0 or pH 9.5. At pH 10.0, the same amount of enzyme converted 80% of the substrate. This is consistent with the decrease of specific activity of NitEg at pH 10.0 [14]. Therefore, the enzyme load was increased to 18.0 μg/mL in the reaction at pH 10.0, which resulted in an about 95% conversion (Fig. 4).

Next, NitEg and BceAmiF were used in a "one-pot" reaction with 5 mM fCN as substrate, and fCN and the reaction products were monitored. The reactions were performed at pH 9.0, 9.5 and 10.0 (Fig. 5). The amount of NitEg was increased in comparison with the single step reactions to reduce the time needed for fCN removal and thus alleviate the negative effect of fCN on BceAmiF. Thus, the cascade reactions were carried out with 38.5 μg NitEg per mL at all pH values, while the amount of BceAmiF was 19.5 μg in all runs. In all buffers an almost complete removal of 5 mM fCN was achieved. Formic acid was determined by a kit based on formate dehydrogenase (EC 1.17.1.9; FDH) converting formic acid to carbon dioxide with the concomitant production of NADH. After a 5-min reaction, the concentrations of formic acid were 5.0 ± 0.3 mM, 5.3 ± 0.3 mM and 3.9 ± 0.4 mM at pH 9.0, 9.5 and 10.0, respectively. After a 10-min reaction, formic acid was found in concentrations of 4.6 ± 0.2 mM, 5.3 ± 0.4 mM and 4.8 ± 0.1 mM at pH 9.0, 9.5 and 10.0, respectively. No significant amount of formamide was found in the reaction mixtures.

The cascade described here (Fig. 6A) differs in several features from the one described before (Fig. 6B). The previous cascade consisted of bench-scale columns filled with immobilized catalyst and was operated

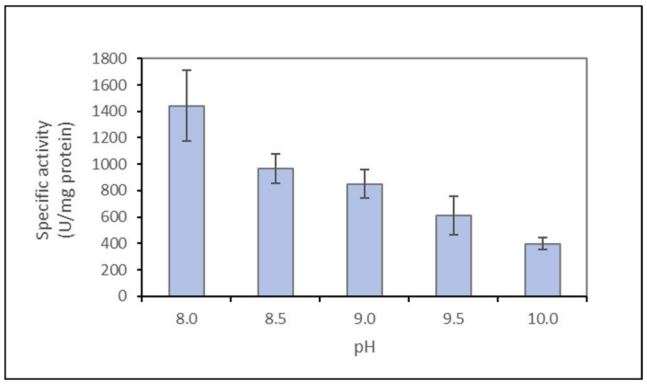

Fig. 1. Effect of pH on the specific activity of purified formamidase BceAmiF from *Bacillus cereus*. The activity was determined using reaction mixtures (0.5 mL) containing Britton-Robinson buffers, pH 8.0–10.0, 0.2–1.1 μg enzyme and 100 mM formamide. The reactions proceeded at 30 °C for 15 min and the residual formamide was determined photometrically [14].

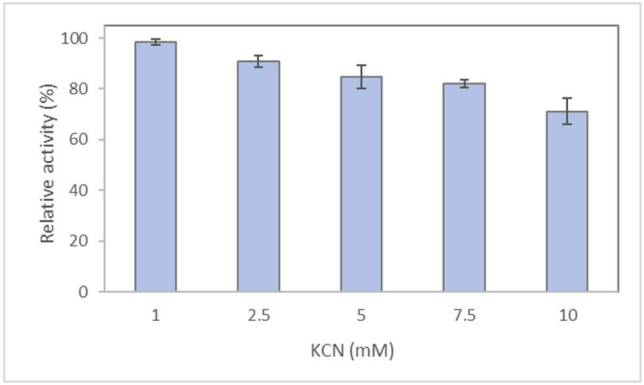

Fig. 2. Effect of KCN on the specific activity of purified formamidase BceAmiF from *Bacillus cereus*. The activity was determined using reaction mixtures (0.5 mL) containing Britton-Robinson buffer, pH 9.0–10.0, 2.2 μg enzyme and 100 mM formamide. The reactions proceeded at 30 °C for 15 min and the residual formamide was determined photometrically [14].

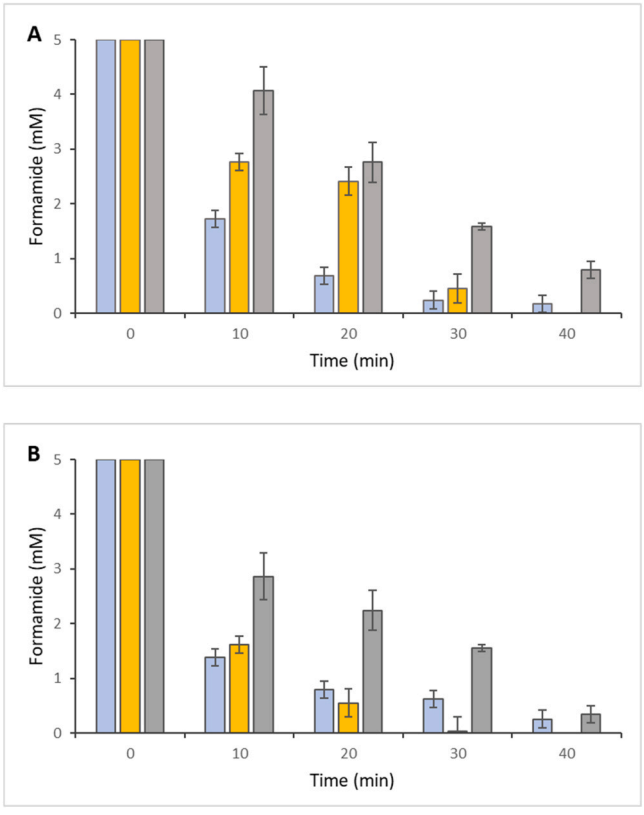

Fig. 3. Conversion of formamide by purified formamidase BceAmiF from *Bacillus cereus*. The reaction mixtures (total volume 0.5 mL) contained 100 mM glycine/NaOH buffer, the purified enzyme and 5 mM formamide. The reaction proceeded with (A) 7.3 μg or (B) 14.6 μg of enzyme per mL at ( ) pH 9.0, ( ) pH 9.5 or ( ) pH 10.0 at 30 °C and 350 rpm. The residual formamide was determined photometrically [14].

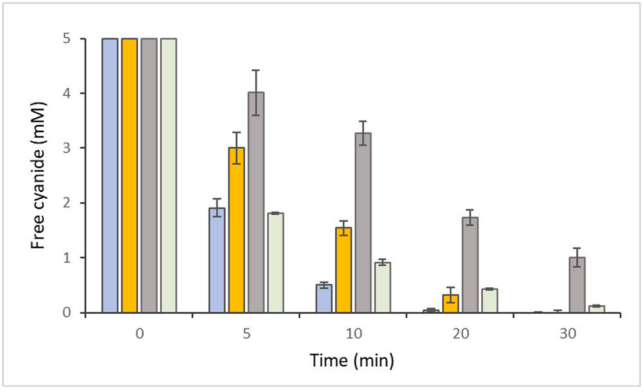

Fig. 4. Conversion of free cyanide by purified cyanide hydratase NitEg from *Exidia glandulosa*. The reaction mixtures (total volume 1 mL) contained 100 mM glycine/NaOH buffer, the purified enzyme and 5 mM KCN. The reaction proceeded with at ( ) pH 9.0, ( ) pH 9.5 or ( ) pH 10.0 with 5.8 μg of enzyme per mL, or at ( ) pH 10.0 with 18.0 μg of enzyme per mL at 30 °C and 350 rpm. The residual cyanide was determined photometrically [14].

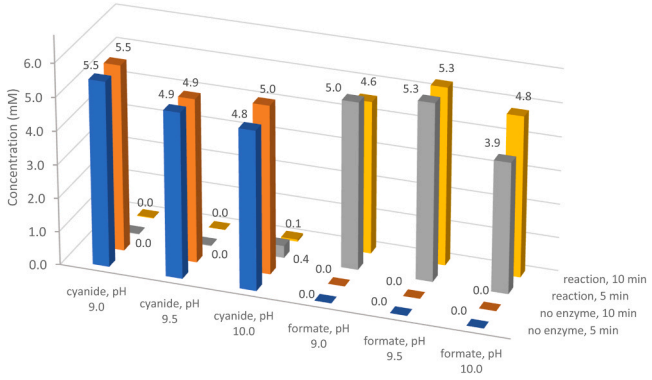

Fig. 5. Conversion of free cyanide (5 mM) to formic acid by a cascade reaction catalyzed by cyanide hydratase NitEg from *Exidia glandulosa* and formamidase BceAmiF from *Bacillus cereus*. The reaction mixtures (total volume 2 mL) contained 100 mM glycine/NaOH buffer, pH 9.0, 9.5 or 10.0, purified NitEg (38.5 μg/mL), purified BceAmiF (19.5 μg/mL) per mL and 5 mM KCN. The reaction proceeded at 30 °C and 350 rpm. Free cyanide and formamide were determined photometrically [14]. Only traces of formamide were found (*not shown*). Formic acid was determined photometrically using a Formic Acid Assay Kit (Megazyme).

in continuous mode. The two catalysts were separated from each other (in two columns or a single column with two zones) [19]. This was found to be not necessary in our ("one-pot") system based on purified enzymes, as the second reaction was not significantly inhibited by cyanide unlike in the case of the whole cells used previously. In addition, almost no residual formamide was found in our system, while the previous system left some formamide (≈16%) unreacted. On the other hand, the continuous operation of the previous system is a benefit. The two-column system was stable for 26 days [19]. Therefore, we will continue our study with the immobilization of NitEg and BceAmiF to allow continuous operation.

The cost of the catalyst can be significant if purified enzymes are used, even if the specific activities of the enzymes are extremely high as in this work. Therefore, the enzyme amounts needed for the cascade reaction will be optimized. In addition, immobilization will reduce the consumption of the enzymes. However, it seems more likely that the enzymes will be used in the future for the detoxification of small quantities of wastewater or for small-scale analytical applications than for the remediation of large amounts of effluents.

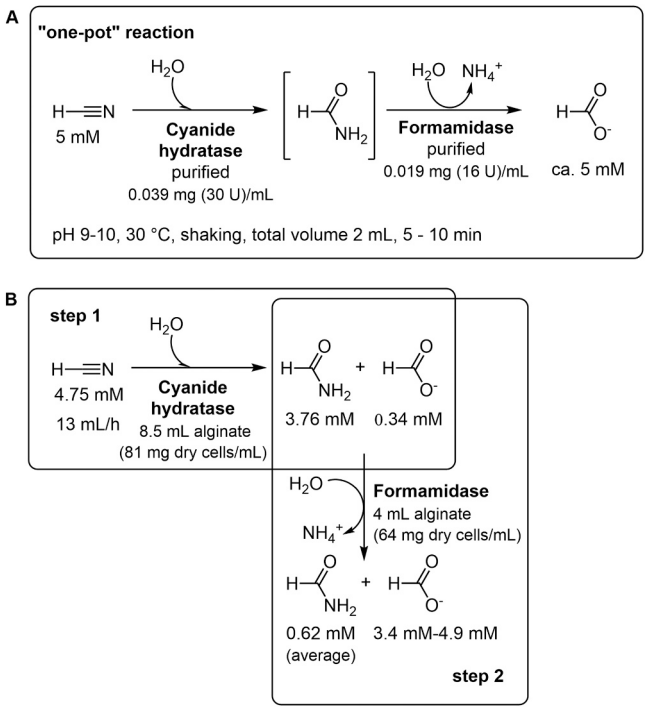

Fig. 6. Degradation of free cyanide to formic acid by cyanide hydratase – amidase cascade reactions. (A) A two-step "one-pot" process described in this work. The catalysts were purified enzymes – cyanide hydratase NitEg from *Exidia glandulosa* and formamidase BceAmiF from *Bacillus cereus*. The process was carried out in batch mode. (B) A two-step sequential process described previously [19]. The catalysts were whole cells of *Fusarium oxysporum* and *Methylobacterium* sp. with cyanide hydratase and formamidase activity, respectively. The process was carried out in flow mode.

4. Conclusions

In this work, we have demonstrated an efficient CynH–AmiF cascade that can be used for the detoxification of fCN. The range of characterized CynH candidates was larger compared with AmiFs although the sequence space of hypothetical AmiF enzymes was found to be broad. The selected CynH and AmiF enzymes were heterologously produced, and their activities were found to be excellent. The storage stability of NitEg and BceAmiF is very good. We investigated whether these enzymes were also compatible with each other in terms of their pH profiles, and this was confirmed. Hence, the cascade consisting of these two enzymes efficiently degraded fCN to relatively benign and biodegradable products (formic acid, ammonia) without leaving significant levels of formamide which still exhibits toxic effects. The rapid removal of fCN, the efficiency of formamide elimination, and the "one-pot" operation mode of our system are advantageous. The next step will be to immobilize the purified enzymes and investigate their continuous use and/or recycling. The observation that the two-enzyme cascade converts fCN to an almost equimolar amount of formic acid suggests the possibility of using the enzymes together with FDH for fCN determination based on NADH monitoring. We are currently investigating this possibility.

CRediT authorship contribution statement

Barbora Křístková: Writing – original draft, Investigation. **Michael Kotik:** Writing – review & editing, Investigation. **Petr Novotný:** Writing – review & editing, Methodology, Investigation. **Katarína Šťastná:** Writing – review & editing, Methodology, Investigation. **Pavla Bojarová:** Writing – review & editing, Project administration, Funding acquisition. **Romana Příhodová:** Investigation. **Miroslav Pátek:** Writing – review & editing. **Ludmila Martínková:** Writing – review &

editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Lenka Rucká:** Writing – review & editing, Investigation. **Natalia Kulik:** Writing – review & editing, Writing – original draft, Investigation.

Declaration of Competing Interest

All authors declare that no competing interests exist.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.procbio.2024.04.009.

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