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Purification and characterization of a nitrilase from *Fusarium solani* O1

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Abstract

An intracellular nitrilase was purified from a *Fusarium solani* O1 culture, in which the enzyme (up to 3000 U L^{-1}) was induced by 2-cyanopyridine. SDS-PAGE revealed one major band corresponding to a molecular weight of approximately 40 kDa. Peptide mass fingerprinting suggested a high similarity of the protein with the putative nitrilase from *Gibberella moniliformis*. Electron microscopy revealed that the enzyme molecules associated into extended rods. The enzyme showed high specific activities towards benzonitrile (156 U mg^{-1}) and 4-cyanopyridine (203 U mg^{-1}). Other aromatic nitriles (3-chlorobenzonitrile, 3-hydroxybenzonitrile) also served as good substrates for the enzyme. The rates of hydrolysis of aliphatic nitriles (methacrylonitrile, propionitrile, butyronitrile, valeronitrile) were 14–26% of that of benzonitrile. The nitrilase was active within pH 5–10 and at up to 50 °C with optima at pH 8.0 and 40–45 °C. Its activity was strongly inhibited by Hg^{2+} and Ag^{+} ions. More than half of the enzyme activity was preserved at up to 50% of *n*-hexane or *n*-heptane or at up to 15% of xylene or ethanol. Operational stability of the enzyme was examined by the conversion of 45 mM 4-cyanopyridine in a continuous and stirred ultrafiltration-membrane reactor. The nitrilase half-life was 277 and 10.5 h at 35 and 45 °C, respectively.

Keywords: Nitrilase; *Fusarium solani*; Nitrile hydrolysis; Substrate specificity; Enzymatic properties

1. Introduction

Nitrilases became widely acknowledged as mild and environment-friendly biocatalysts for nitrile hydrolysis. A number of bacterial nitrilases with different substrate specificities were characterized within the past 20 years (see Ref. [1] for review). Recently, we became interested in filamentous fungi as an alternative source of nitrilases with potential use in biocatalysis. Previous works suggested occurrence of these enzymes in the genera of *Fusarium*, *Gibberella*, *Aspergillus* and *Penicillium* [2–5]. In addition, genes encoding putative nitrilases in *Aspergillus fumigatus*, *Aspergillus nidulans*, *Gibberella moniliformis*, *Gibberella zeae*, *Neosartorya fischeri* etc. were published in sequence databases (see http://www.ncbi.nlm.nih.gov). Nevertheless, only two nitrilases (from *Fusarium solani* IMI 196840 [6] and *Fusarium oxysporum* f. sp. *melonis* [7]) were characterized in some detail. However, no data were published regarding the amino acid sequences of these enzymes.

The scarceness of data on fungal nitrilases inspired us to examine the occurrence and properties of these enzymes. A screening for isolates utilizing 3-cyanopyridine as sole nitrogen source resulted in identification of *Aspergillus niger*, *F. solani* and *Penicillium multicolor* as perspective nitrilase producers [8]. Hyperinduction by 2-cyanopyridine increased the nitrilase production by up to three orders of magnitude in comparison with 3-cyanopyridine [9] and a nitrilase was partially purified from hyperinduced cells of *A. niger* [10]. The nitrilases from filamentous fungi [6–8,10] seem to share common enzymatic properties

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like high specific activities for aromatic and heterocyclic nitriles and a good thermal stability. These qualities make them perspective biocatalysts for the mild hydrolysis of (hetero)aromatic nitriles.

In the present work, we focused on enzymatic properties of a new *F. solani* nitrilase. Examination of this enzyme using whole cells and cell-free extracts demonstrated its biocatalytic utility [8,9,11,12]. This encouraged us to purify and characterize the enzyme, which proved to be different from a previously reported nitrilase produced by the same species [6] in terms of subunit molecular weight, substrate specificity and specific activity.

2. Experimental

2.1. Materials

Substrates and authentic standards of reaction products were purchased from Sigma–Aldrich or Merck. Chemicals for protein sequencing were purchased from Applied BioSystems. Columns for protein purification were supplied by GE Healthcare.

2.2. Microorganism and cultivation

F. solani O1 (deposited in the Culture Collection of Fungi, Charles University Prague, Czech Republic; accession number CCF 3635) was grown in a two-stage culture as described previously [11] with slight modifications (increased medium volume – 200 mL/500 mL Erlenmeyer flask – and increased 2-cyanopyridine concentration: 3 g L^{-1}).

2.3. Cell lyophilization

The cells were frozen at −80 °C, lyophilized overnight and stored at −20 °C until further use.

2.4. Enzyme purification

The enzyme was purified from a cell-free extract, which was obtained by grinding the lyophilized mycelium with a pestle in a mortar, resuspending 1 g of the homogenate in 15–20 mL of extraction buffer (50 mM Tris/HCl, pH 8.0; 0.8 M ammonium sulfate) and sonication in an ultrasonic bath (2 min × 5 min, 35 kHz, ELMA, Germany). After each sonication the suspension was stirred at 4 °C for 10 min. Cell debris was removed by centrifugation (13,000 × *g*, 4 °C, 30 min). The extract was twice diluted with the extraction buffer and applied to a Hi-Prep 16/10 Phenyl FF column (low sub) pre-equilibrated with the same buffer. Proteins were eluted with a linear gradient of ammonium sulfate (0.8–0 M, 60 mL) in Tris/HCl buffer (50 mM, pH 8) at a flow rate of 2 mL min^{-1}. Nitrilase eluted at 520–200 mM ammonium sulfate. Active fractions were pooled, concentrated using an Amicon Ultra-4 unit (Millipore), applied to a 16/60 Hi-Prep Sephacryl S-200 column pre-equilibrated with Tris/HCl buffer (50 mM, pH 8.0; 150 mM NaCl) and eluted with the same buffer at a flow rate of 0.5 mL min^{-1}. The active fractions were pooled, concentrated, 5 times diluted with 50 mM Tris/HCl buffer, pH 8.0, and applied to a Hi-Prep 16/10 Q FF column. Proteins were eluted with a linear gradient of NaCl (0–1 M) in Tris/HCl buffer (50 mM, pH 8.0) at a flow rate of 2 mL min^{-1}. Nitrilase eluted at 300–450 mM NaCl. Active fractions were pooled, concentrated and sucrose was added to a concentration of 2%. The purified enzyme was stored at −80 °C.

2.5. Protein assay

Protein was determined according to Bradford [13] using bovine serum albumin as the standard.

2.6. Gel filtration on Superdex 200

The purified enzyme was applied to a Superdex 200 HR 10/300 GL column pre-equilibrated with Tris/HCl buffer (50 mM, pH 8.0; 150 mM NaCl). Protein was eluted with the same buffer at a flow rate of 0.5 mL min^{-1}. The column was calibrated in the range of 158–669 kDa using a high molecular weight calibration kit (GE Healthcare).

2.7. SDS-PAGE

SDS polyacrylamide gel electrophoresis was performed according to Laemmli [14] in 12% polyacrylamide slab gels. The marker proteins were protein molecular weight standards in the range of 14.4–97 kDa (GE Healthcare).

2.8. Determination of the N-terminal amino acid sequence

The N-terminal sequence was analyzed as described for the nitrilase from *A. niger* K10 [10].

2.9. Mass spectrometry analysis

Peptide mass fingerprinting of fragments obtained by tryptic digestion of the enzyme was carried out as described for the nitrilase from *A. niger* K10 [10]. Alternatively, peptide fragments were obtained by Asp-N protease digestion and analyzed in the same way. Homologous proteins were searched in the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.10. Assay for nitrilase activity

The nitrilase activity was assayed with 25 mM benzonitrile (from 500 mM stock solution in methanol) in 50 mM Tris/HCl (pH 8.0) at 45 °C. The reaction was started by the addition of substrate after 5-min preincubation at 45 °C and quenched after 10 min with 1 M HCl (0.01 mL/0.1 mL of the reaction mixture). The substrate specificity was assayed with 25 mM nitriles under the same conditions. Kinetic values for benzonitrile, 3-cyanopyridine and 4-cyanopyridine were determined within the substrate concentration range of 1–25 mM. Optimum pH was determined using 50 mM Britton-Robinson (acetic acid/boric acid/phosphoric acid/NaOH) buffers (pH 4–12) or 50 mM Tris/HCl buffers (pH 7–9) at 45 °C. Optimum temperature was determined with reactions performed at pH 8.0 (50 mM

Tris/HCl buffer) and different temperatures (35–60 °C). Concentrations of substrates and products were determined by HPLC as described below.

Alternatively, rapid semiquantitative detection of the nitrilase activity in fractions after purification steps was performed by continuously monitoring the absorption of benzoic acid at 238 nm with $\varepsilon = 3.3\ \mathrm{L\ mmol^{-1}\ cm^{-1}}$ on Shimadzu PharmaSpec UV-1700. The reaction proceeded in Suprasil Quartz cuvettes (volume 700 μL, optical length 10 mm, Hellma) containing 690 μL of 0.5 mM benzonitrile in Tris/HCl (50 mM, pH 8) and 10 μL of the enzyme solution at 45 °C. A reaction mixture without enzyme was used as the blank.

2.11. Effect of inhibitors and cosolvents

The activity of the enzyme was assayed at standard conditions (see above) in reaction mixtures containing different metal ions, cosolvents or other additives.

2.12. Electron microscopy

Enzyme complexes in 50 mM Tris/HCl buffer, pH 8 (~87 μg of protein $\mathrm{mL^{-1}}$) were let to adsorb onto glow-discharge activated thin carbon support film [15] on 400 mesh copper grids for 30 s. Then the rest of the solution remaining on the grids was sucked out with filter paper and the grids were negatively stained with 2% uranyl acetate in double distilled water for 30 s. Excess staining solution was blotted off with filter paper. The grids were air-dried and examined in a Philips CM100 electron microscope (FEI, formerly Philips EO, The Netherlands) equipped with MegaView*II* (Soft Imaging Systems, GmbH) slow scan digital camera. The images were digitally recorded at 80 kV and at primary magnification of 92,000×, which gives 0.7 nm of pixel size.

2.13. Biotransformations in an ultrafiltration membrane reactor

An ultrafiltration cell (UF-cell), Amicon model 8050 (Millipore, USA) with 72-mL fluid volume was equipped with polyethersulfone ultrafiltration membrane (molecular weight cut-off 30 kDa) from Amicon. The volumetric flow ($7.3 \pm 0.1\ \mathrm{mL\ h^{-1}}$) was controlled using a peristaltic pump *P*-1 (GE Healthcare). The system was continuously stirred at 150 rpm. The reactor was submerged in a thermostated water bath, the temperature of which (35 or 45 °C) was controlled within 0.1 °C. The feed stream contained 45 mM of 4-cyanopyridine dissolved in Tris/HCl buffer (50 mM, pH 8.0). The effluent stream containing substrate and products was collected in a fraction collector and analyzed by HPLC as described below. The operational deactivation constant ($\mathrm{h^{-1}}$) and initial reaction rate ($\mu\mathrm{mol\ min^{-1}\ mg_{protein}{}^{-1}}$) were calculated as described previously [16].

2.14. Analytical HPLC

Nitriles, amides and acids were analyzed by HPLC with a Novapak C_{18} column (4 μm, 150 mm × 3.9 mm; Waters). 3-Cyanopyridine, 4-cyanopyridine and the corresponding carboxylic acids and amides were separated using a mobile phase consisting of 4% (v/v) acetonitrile in 5 mM Na_2HPO_4/H_3PO_4 buffer, pH 7.2, at a flow rate of $0.9\ \mathrm{mL\ min^{-1}}$ and 35 °C. Other compounds were separated as described previously [10,17].

3. Results and discussion

3.1. Purification and structure of the nitrilase from F. solani O1

The soil isolate *F. solani* O1 formed an intracellular nitrilase at levels of $\geq 3000\ \mathrm{U\ L^{-1}}$ of culture. Minor modifications of the two-stage cultivation protocol [11] (increase of medium volume and 2-cyanopyridine concentration) enhanced the total enzyme production by about one third. The nitrilase production/L of *F. oxysporum* f. sp. *melonis* culture was considerably lower (830 U at maximum) [7]. In case of *F. solani* IMI 196840 [6], the nitrilase yield was only 59 U from 15 L of culture. Strain O1 also yielded much higher nitrilase levels than *A. niger* K10 (approximately $160\ \mathrm{U\ L^{-1}}$ of culture). Therefore, strain O1 appeared to be the most efficient nitrilase producer reported among filamentous fungi to date.

Up to 90% of the whole-cell activity was recovered in the cell-free extract, indicating a good stability of the enzyme during the disintegration procedure. It is notable that the specific activity of the nitrilase in cell-free extract increased from 3.9 [11] up to $15.7\ \mathrm{U\ mg_{protein}{}^{-1}}$. This was mainly due to the use of an extraction buffer supplemented with 0.8 M ammonium sulfate. In this way, a large ratio of contaminating cellular protein was removed, while the nitrilase was left in solution.

The enzyme was purified nearly 10 times. Approximately 26% of the original activity was recovered, which corresponded to $5.2\ \mathrm{mg_{protein}}$ (see Table 1). SDS-PAGE revealed one major band with a molecular mass of approximately 40 kDa (see

Table 1
Purification of a nitrilase from *Fusarium solani* O1

Step	Total protein (mg)	Specific activity ($\mathrm{U\ mg^{-1}}$)	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	199.2	15.7	3125	100	1
Hydrophobic chromatography on Phenyl Sepharose	36.0	28.5	1026	32.8	1.8
Gel filtration on Sephacryl S-200	14.7	67.0	985	31.5	4.3
Anion exchange on Q-Sepharose	5.2	156.0	811	25.9	9.9

Note: the enzyme activity was assayed with 25 mM benzonitrile (see Section 2 for details).

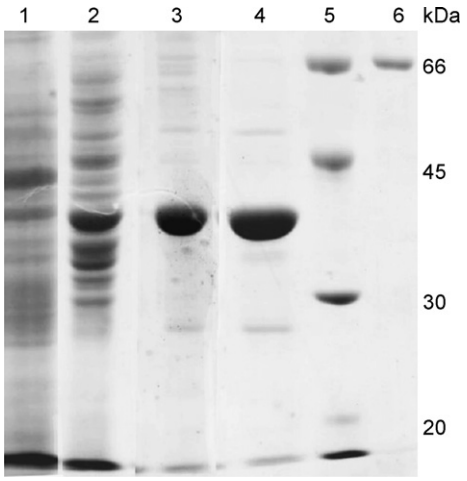

Fig. 1. SDS-PAGE of nitrilase samples stained with Coomassie Brilliant Blue R-250, lane 1: cell-free extract, lane 2: after Phenyl Sepharose, lane 3: after Sephacryl S-200, lane 4: purified enzyme (concentrated after Q Sepharose), lane 5: markers (bovine serum albumin 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 20.1 kDa), lane 6: bovine serum albumin.

Fig. 1). N-terminal sequencing and mass spectrometry analysis showed that four peptide fragments (including the N-terminus) of this protein were identical with the corresponding amino acid sequences of a putative nitrilase from *G. moniliformis* (see Fig. 2). The apparent molecular weight of the new nitrilase is in accordance with those of other nitrilases from bacteria and *F. oxysporum* f. sp. *melonis* having subunits of 32–45 kDa (see Ref. [1] for review). On the other hand, the nitrilase from *F. solani* with a subunit of 76 kDa seems to be exceptional [6]. According to gel filtration the molecular weight of the holoenzyme was about 580 kDa. This indicates its multimeric structure, which was also reported for most other nitrilases (see Ref. [1] for review).

The purified enzyme (1.65 $mg_{protein}$ mL^{-1}) was very stable when stored at −80 °C in presence of 2% (w/v) sucrose as a stabilizer. No significant activity loss was observed during 2 months under these conditions. After five freeze-thaw cycles (one cycle per day), the same nitrilase sample stored at −18 °C with sucrose (2%, w/v) lost only 8% of its initial activity.

3.2. Electron microscopic study of enzyme structure

Examination of the purified nitrilase by electron microscopy revealed formation of extended complex rods, up to 500 nm in length and approximately 24 nm thick, or aggregates (see Fig. 3A and B). The negative staining was done according to standard procedure, however, we cannot exclude that rod formation and their aggregation could occur during the sample preparation. The high activity yield in gel filtration (see Table 1), where the enzyme was eluted as a single peak corresponding to 580 kDa, suggests that aggregation could take place after enzyme purification or during negative staining procedure. Similar structures were previously reported for the cyanide dihydratase from *B. pumilus* [18]. The resemblance in quaternary structure of both enzymes is in contradiction to the significant difference in amino acid sequences of the nitrilase of *G. moniliformis*, which appears to be highly homologous to the enzyme from *F. solani*, and cyanide dihydratases from *B. pumilus* (only 30% homology). Therefore, we became interested in the quarternary structure of another fungal nitrilase, which was recently semi-purified from *A. niger* K10 [10]. As judged from its N-terminal amino acid sequence [10], this enzyme is highly homologous with a putative nitrilase of *A. fumigatus*, which, in turn, exhibits a low homology (about 30%) both to the nitrilase of *G. moniliformis* and the cyanide dihydratases from *B. pumilus*. The nitrilase from *A. niger* also formed long rods 12 ± 1 nm in thickness reaching up to 250 nm,

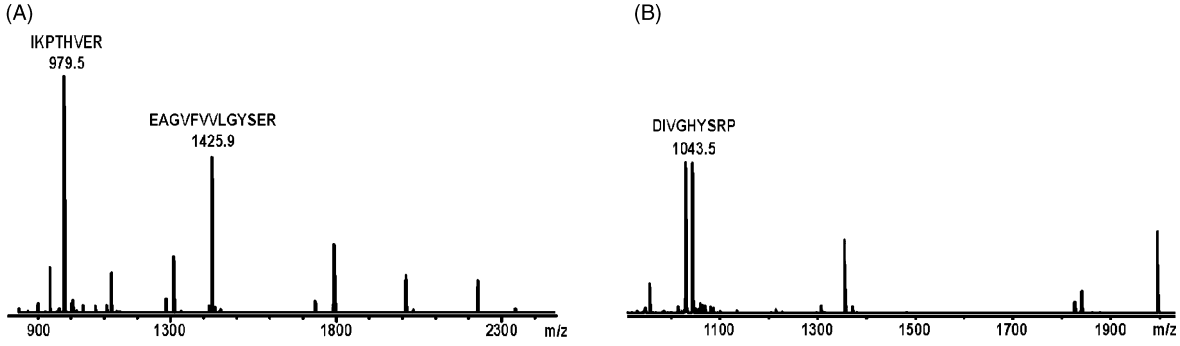

Fig. 2. Mass spectrometry analysis of nitrilase from *Fusarium solani*. (A and B) MALDI mass spectra of peptides extracted after in-gel digestion of nitrilase separated by SDS polyacrylamide gel electrophoresis using trypsin and Asp-N protease, respectively. Only those peptides, for which the complete sequence ladder obtained after post-source decay fragmentation could be recorded, are indicated. (C) The peptide sequences obtained by automated Edman degradation of PVDF blotted nitrilase (underlined), or by mass spectrometry of digests after trypsin (underlined and bold) or Asp-N (underlined and italics) matched exactly three peptide sequences obtained by translation of the gene for the putative nitrilase from *Gibberella moniliformis* (anamorph: *Fusarium verticillioides*) found in the GeneBank using BLAST algoritm (accession number DQ534528.1). Note the poor cleavage of the Lys-Pro bond in the shorter tryptic sequence IKPTHVER.

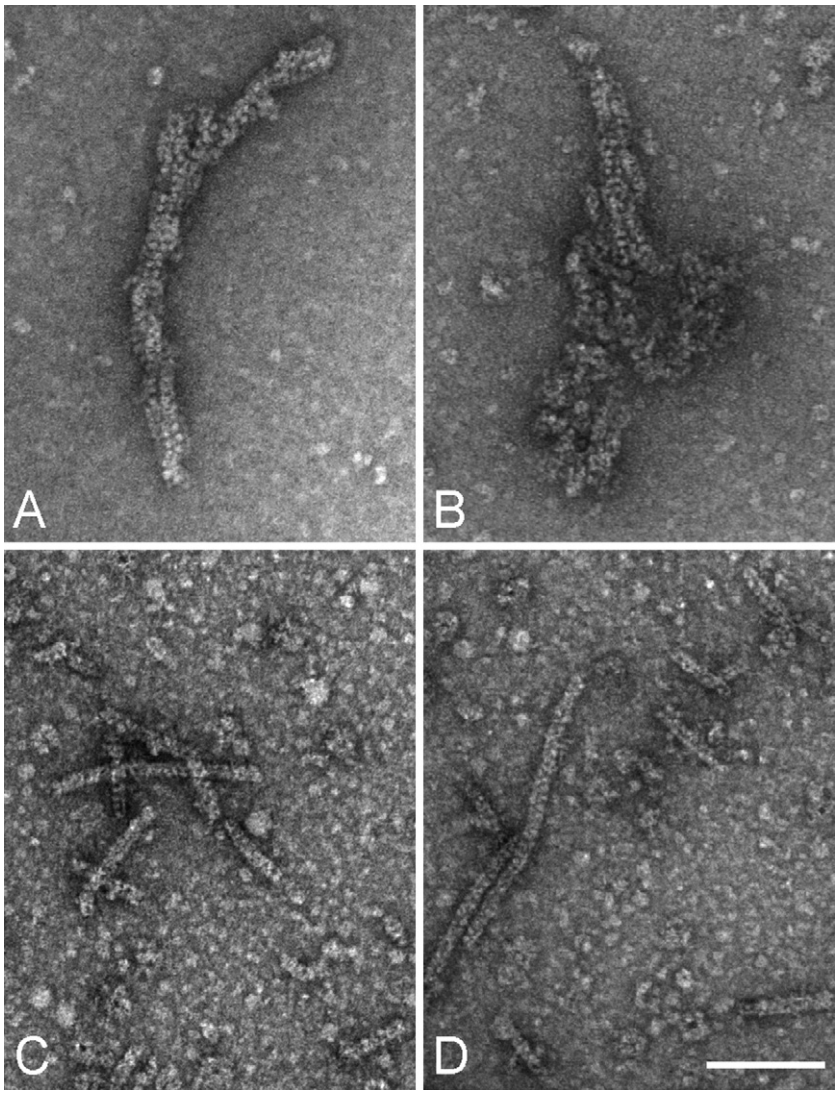

Fig. 3. Electron micrographs of negatively stained purified nitrilases from *F. solani* (A and B) and *A. niger* K10 (C and D). Scale bar represents 100 nm. See Section 2 for details.

which, however, were distinct in structure from those of *F. solani* nitrilase (see Fig. 3C and D). The formation of aggregates in samples of nitrilase from *A. niger* was not observed.

3.3. Effect of inhibitors

Nitrilases, as enzymes that are supposed to harbor a catalytically active cystein [1], are expected to be sensitive towards organic sulfhydryl reagents and heavy metal ions reacting with sulfhydryl groups. The present results were consistent with this expectation (see Table 2). From metal ions, the enzyme was most severely inhibited by Hg^{2+} and Ag^{+} (at 1 mM), but Cu^{2+} (at 1 mM), Al^{3+}, Fe^{3+}, Zn^{2+}, Pb^{2+} and Ni^{2+} (all at 5 mM) also proved to be very effective inhibitors. Inhibition by silver, cupric and mercuric ions and trivalent ions was in compliance with the results obtained for the nitrilase from *F. oxysporum* f. sp. *melonis* [7]. However, a difference was observed in the effect of divalent ions, which stabilized the enzyme from *F. oxysporum* f. sp. *melonis* [7] but partially inhibited the enzyme from strain O1 (e.g., Ni^{2+}, Co^{2+}). In general the effect of metal ions on the new enzyme was similar as in case of nitrilase from *A. niger* with a few exceptions (more pronounced inhibition of the new enzyme by Ni^{2+} and Fe^{3+}). In general nitrilases were more severely inhibited by *p*-hydroxymercuribenzoate or *p*-chloromercuribenzoate than by iodoacetamide. Possible explanation suggested by Goldlust and Bohak [7] was the poor accessibility of the catalytically active cystein for the reaction with iodoacetamide and other sulfhydryl reagents like iodoacetate and *N*-ethylmaleinimide. Destabilizing or inhibitory effect of dithiothreitol on nitrilases from, e.g., *Pseudomonas fluorescens* [19], *A. niger* K10 [10] or *F. solani* O1 is not uncommon and may be related to the potential of this compound to cleave disulfide bonds in proteins. Metal ion chelators like EDTA showed no pronounced inhibitory effect on nitrilases (enzymes lacking metal cofactors) as also observed for the new enzyme.

3.4. Substrate specificity

The specific activity of the purified nitrilase for benzonitrile was approximately 156 U $mg_{protein}{}^{-1}$. This is a 94-times higher

Table 2
Effect of different ions and other reagents on nitrilase activity

Compound (mM)	Residual activity (%)
None	100
$AgNO_3$ (1)	1.5
$Al_2(SO_4)_3$ (5)	0.3
$CoCl_2$ (5)	46.3
$CuSO_4$ (1)	13.4
$Cr_2(SO_4)_3$ (1)	104
$FeCl_3$ (5)	5
$HgCl_2$ (1)	0
$MgSO_4$ (5)	46
$NiSO_4$ (5)	7
$Pb(NO_3)_2$ (1)	6
$ZnSO_4$ (5)	6
EDTA (1)	87
Dithiothreitol (1)	55
Iodoacetamide (4.6)	5
p-Hydroxymercuribenzoate (1)	12
NaN_3 (5)	38

Note: the nitrilase activity was assayed with 25 mM benzonitrile (see Section 2 for details). The activity of the enzyme in absence of additives (156 U mg^{-1}) was taken as 100%.

activity than reported for the nitrilase from another strain of *F. solani* [6] but comparable with the specific activity of the purified nitrilase from *F. oxysporum* f. sp. *melonis* (143 U $mg_{protein}{}^{-1}$) [7].

Determination of the substrate specificity showed that the new enzyme was an aromatic nitrilase (see Table 3), as already indicated by whole-cell experiments [8,9,11]. From the substrates tested, the enzyme exhibited the highest specific activity for 4-cyanopyridine (203 U $mg_{protein}{}^{-1}$) as the nitrilase from *A. niger* [10]. The enzyme was also useful for hydrolysis of 3-cyanopyridine, *m*- and *p*-substituted aromatic nitriles and unbranched aliphatic nitriles. The poorly soluble 1,3- and 1,4-dicyanobenzene also served as acceptable substrates (data not shown).

Table 3
Substrate specificity of the purified nitrilase

Substrate	Relative activity [%]	K_M (mM)
Benzonitrile	100	1.5
3-Hydroxybenzonitrile	80	
4-Hydroxybenzonitrile	3	
4-Aminobenzonitrile	7	
3-Tolunitrile	33	
4-Tolunitrile	16	
3-Chlorobenzonitrile	87	
4-Chlorobenzonitrile	40	
3-Cyanopyridine	28	3.2
4-Cyanopyridine	130	1.4
Propionitrile	18	
Butyronitrile	20	
Isobutyronitrile	4	
Valeronitrile	26	
Methacrylonitrile	14	
Lactonitrile	4	

Note: the nitrilase activity was assayed with 25 mM substrates (see Section 2 for details). The specific activity for benzonitrile (156 U mg^{-1}) was taken as 100%.

The nitrilases from *F. solani* O1 and *F. solani* IMI 196840 [6] differed markedly in relative activities towards aromatic nitriles (e.g., 40 and 85.6% for 4-chlorobenzonitrile, 87 and 40.4% for 3-chlorobenzonitrile, 80 and 21% for 3-hydroxybenzonitrile, respectively, if reaction rates of both enzymes for benzonitrile (V_{max} in case of the latter strain) were taken as 100%). Activity towards aliphatic nitriles was not reported for the latter enzyme. The relative activities towards aliphatic nitriles were similar for the new nitrilase and the nitrilase from *F. oxysporum* f. sp. *melonis* [7]. This enzyme, however, showed no activity for 3-hydroxybenzonitrile (a good substrate of *F. solani* O1 nitrilase). Detailed comparison was not possible, as activities of the enzyme from *F. oxysporum* were reported neither for most of the substituted aromatic nitriles examined by us, nor for 4-cyanopyridine.

Branched nitriles (lactonitrile, isobutyronitrile) were poor substrates of the nitrilase, being hydrolyzed at a 4% relative rate in comparison with benzonitrile (see Table 3). However, considerable amount of products could be obtained from these and other branched nitriles (2-chloropropionitrile, 2-phenylpropionitrile, 2-methyl-3-butenenitrile) after extended reaction times (1–3 days at room temperature; data not shown). Examination of the enantiomeric purity of products obtained from the above nitriles (racemates) is in progress.

3.5. Chemoselectivity

At optimum reaction conditions, the product formed by the new nitrilase consisted of <1% of amide in case of benzonitrile conversion and 1–3% of amide in case of 3- and 4-cyanopyridine conversion. At suboptimal conditions (e.g., pH 5.0), the ratio of benzamide in the total product of benzonitrile hydrolysis increased slightly (to ≤2%). Thus it is evident that the new nitrilase produced much less by-products, amides, than the enzymes from *A. niger* K10 [10], *P. fluorescens* EBC 191 [19,20] or *Arabidopsis thaliana* [21], which formed amides as prevailing products from some nitriles, but also less than the nitrilase from *F. oxysporum* [7], which formed 4–6% benzamide from benzonitrile. Therefore, the high chemoselectivity of the new enzyme appears to be a significant advantage whenever hydrolysis of nitriles into pure carboxylic acids is desired. The purity of the carboxylic acids can be further increased by supplementing the reaction mixture with an amidase of suitable substrate specificity, as demonstrated by us recently [12].

It is evident from a recent study on a recombinant nitrilase from *P. fluorescens* EBC 191 [20] that the acid: amide ratio is largely influenced by electron effects as well as by the absolute configuration of substituents at the α-position. A mechanism for the amide formation was hypothesized, proposing destabilization of the positive charge on the nitrogen atom of the CN-group, this charge being essential for interaction with the Glu residue of the Glu-Lys-Cys catalytic triad. It is also obvious from the comparison of different nitrilases that not only the substrate structure, but the structure of the enzyme too is crucial for the mechanism of nitrile conversion by nitrilases. In this respect there is a great difference between the nitrilase from *F. solani* O1, *F. oxysporum* [7] and a number of bacterial nitrilases, on one hand, and the

nitrilases from *A. niger* [10], *A. thaliana* [21] and *P. fluorescens* [19,20], on the other hand. Molecular modelling of nitrilases may be helpful for elucidation of this phenomenon which may be related to different ability in different enzymes to stabilize the positively charged N-atom in the product intermediate.

3.6. Effects of temperature and pH on enzyme activity

The nitrilase from strain O1 exhibited the highest activities at a temperature range between 40 and 45 °C. The activities at 35 and 50 °C were 60 and 26% of the maximum activity, respectively. The enzyme showed only traces of activity at 55 °C. The activity of the enzyme from *F. oxysporum* f. sp. *melonis* was the highest at 40 °C but decreased sharply at 45 °C [6]. However, direct comparison of the temperature optima was not possible due to different reaction times used. On the other hand, the pH optimum of the new enzyme (7–9) was not as broad as that shown for the enzyme from *F. oxysporum* f. sp. *melonis*, which exhibited comparable activities in the range of pH 6–11. The enzyme from strain O1 showed 5, 39 and 29% of the maximum activity at pH 5, 6 and 10, respectively, and it was nearly inactive at pH 11.

3.7. Effect of cosolvents

Since most nitriles are poorly soluble in aqueous media, nitrilases operating in the presence of organic cosolvents are of practical impact. For instance, the recombinant nitrilase from *Synechocystis* sp. PCC6803 showed fair activities in buffer—organic solvent monophasic or biphasic mixtures (particularly in 40% dimethylsulfoxide, 20% methanol or 40% *n*-heptane) [22]. Nitrilase in cell-free extracts from *Pseudomonas* DSM 11397 retained an even higher ratio of its initial activity in the presence of hydrocarbons (95% in 75% *n*-octane, 106% in 95% *n*-hexadecane and 25–58% in buffer-saturated primary alcohols) [23]. The results of benzonitrile biotransformation catalyzed by the nitrilase from strain O1 (see Fig. 4) suggested that this enzyme is also suitable for use in selected organo-aqueous media. More than half of its initial activity was retained in the presence of 5–50% of *n*-hexane or *n*-heptane or 5–15% of xylene or ethanol. In addition, the enzyme showed more than 30% of the control activity with 5–10% of toluene, xylene, dimethylsulfoxide or methanol and at 5% acetonitrile, dichloromethan, 2-propanol, aceton or ethyl acetate. The reaction rate seemed to be also influenced by the distribution of substrate (benzonitrile) between the organic and the aqueous phase (>9: 1 for toluene or xylene, <8: 2 for *n*-hexane or *n*-heptane).

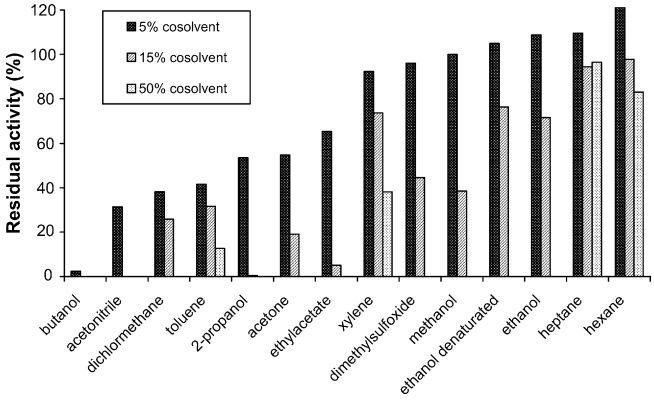

Fig. 4. Effect of organic cosolvents on activity of the purified nitrilase. The nitrilase activity was assayed with 25 mM benzonitrile (see Section 2 for details). The activity of the enzyme in presence of 5% (v/v) methanol (156 U mg^{-1}) was taken as 100%.

3.8. Application of the nitrilase in ultrafiltration membrane bioreactor

The ultrafiltration membrane reactor was shown to be a useful tool for the investigation of enzyme operational stability and for the laboratory-scale studies of continuous biocatalytical processes [16,24]. Using this reactor configuration, bioprocesses for the conversions of, e.g., acrylonitrile or benzonitrile by bacterial cells were proposed (*ibid.*). In this work, nitrilase stability was monitored during the conversion of 4-cyanopyridine, which is superior substrate of this enzyme. Due to the high specific activity of the nitrilase, a very low protein amount (<0.05 mg/reactor) was sufficient to load the 72-mL biore-

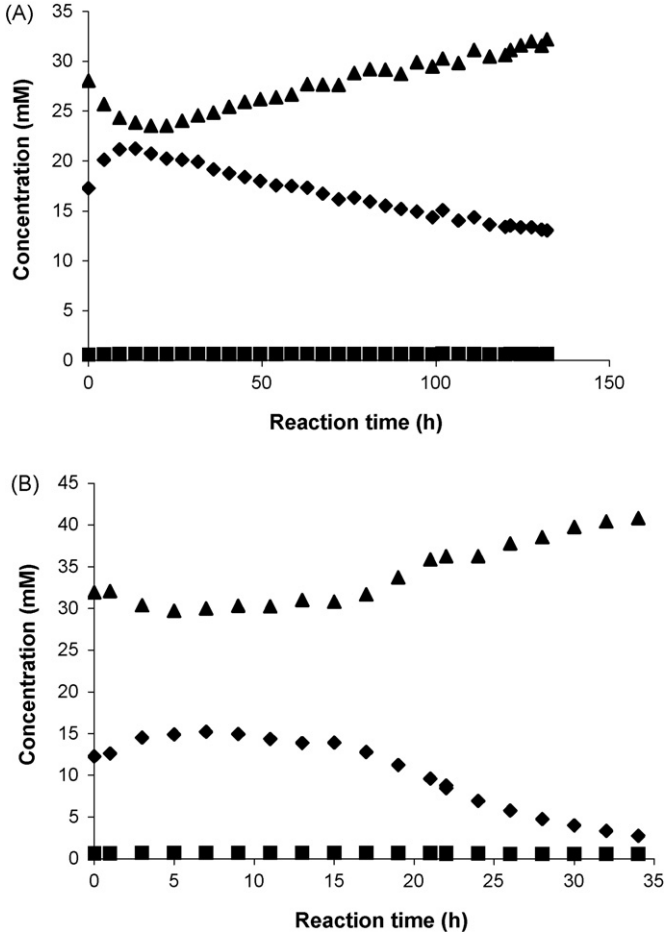

Fig. 5. Conversion of 4-cyanopyridine (45 mM) by the purified nitrilase in an ultrafiltration-membrane reactor at 35 °C (A) and 45 °C (B). (♦) Isonicotinic acid, (■) isonicotinamide, (▲) 4-cyanopyridine. See Section 2 for details.

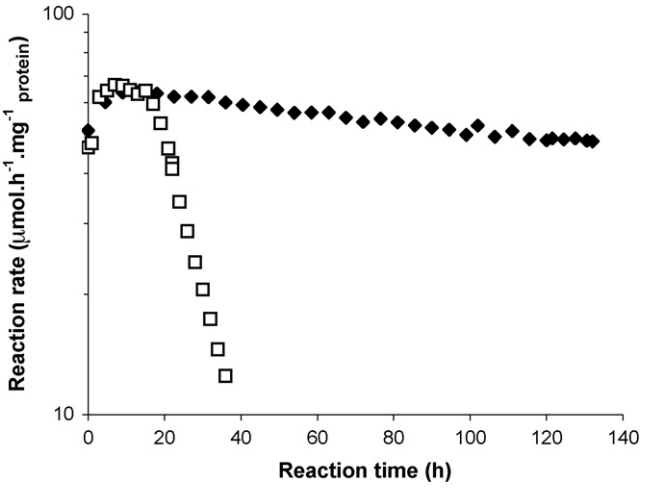

Fig. 6. Effect of temperature on the operational stability of nitrilase in bioconversion of 45 mM 4-cyanopyridine at 35 °C (♦) and (□) 45 °C.

actor. Total conversion was not desired in this experiment, as we aimed at monitoring the changes in the system productivity depending on the reaction conditions (see Fig. 5). Using the above enzyme load, partial conversion of 45 mM 4-cyanopyridine (29–46%) was achieved at 35 °C. Despite the high protein dilution, the enzyme showed a very good stability at this temperature. Under these conditions, the operational deactivation constant and the enzyme half-life was 0.0025 h^{-1} and 277 h, respectively. The linear response in a semilogarithmic plot of reaction rate against process time (see Fig. 6), once the reactor reaches the steady-state, indicates a first-order enzyme deactivation kinetics and the slope of the straight line is the operational deactivation constant. By increasing the operation temperature from 35 to 45 °C, the initial reaction rate, evaluated as ordinate intercept, increased from 65.8 (regression coefficient = 0.97) to 152 (regression coefficient = 0.92) μmol min^{-1} $mg_{protein}{}^{-1}$. However, the deactivation constant increased up to 0.066 h^{-1} and the enzyme half-life decreased to 10.5 h. Nevertheless, operation of the reactor at 45 °C should be practicable as the activity loss can be balanced by increasing the enzyme load, which was very low in the present experiment.

4. Conclusion

The new nitrilase from a soil isolate *F. solani* O1 is available at high levels by cultivation of the strain at hyperinduction conditions. The specific activity of the enzyme exceeds those of the most nitrilases reported to date. The enzyme is highly active towards heterocyclic nitriles important as intermediates of pharmaceuticals (3-cyanopyridine—precursor of nicotinic acid, vitamin; 4-cyanopyridine—precursor of isonicotinic acid hydrazide, tuberculostatic). Chemoselectivity of the enzyme guarantees a high product purity, which, however, can be further increased by using a bienzymatic nitrilase—amidase catalyzed process. These properties, together with the good stability make this enzyme a promising biocatalyst for mild nitrile hydrolysis.

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