Purification and characterization of nitrilase from Fusarium solani IMI196840

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\textbf{ABSTRACT}

Nitrilase activity in \textit{Fusarium solani} IMI196840 (approx. 1500U$^{-1}$ of culture broth) was induced by 2-cyanopyridine. The enzyme was purified by a factor of 20.3 at a yield of 26.9\%. According to gel filtration, the holoenzyme was an approx. 550-kDa homooligomer consisting of subunits with a molecular weight of approximately 40 kDa, as determined by SDS-PAGE. Mass spectrometry analysis of the tryptic fragments suggested a high similarity of this enzyme to the hypothetical CN hydrolases from \textit{Aspergillus oryzae}, \textit{Gibberella zeae}, \textit{Gibberella moniliformis} and \textit{Nectria haematococca}. Circular dichroism and fluorescence spectra indicated that secondary structure content and overall tertiary structure, respectively, were almost identical in nitrilases from \textit{F. solani} IMI196840 and \textit{F. solani} O1. The melting temperatures of the enzymes were 49.3 °C and 47.8 °C, respectively. The best substrates for the purified nitrilase from \textit{F. solani} IMI196840 were benzonitrile and 4-cyanopyridine, which were hydrolyzed at the rates of 144 and 312 U mg$^{-1}$ protein, respectively, under the optimum conditions of pH 8 and 45 °C. The enzyme was highly chemoselective, producing ≤2% amides as by-products.

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1. Introduction

Nitrilases (EC 3.5.5.1; 3.5.5.2; 3.5.5.4–3.5.5.7) have been recognized as valuable biocatalysts for the synthesis of high added-value carboxylic acids from cheap and readily available nitriles. Interest in their use is rising as illustrated by the increasing number of articles and patents on this topic [1]. This is because the enzymes enable nitrile hydrolysis to be performed at mild pH and temperature and are often also enantioselective or regioselective. Furthermore, among nitrilases there is broad choice of enzymes with differing substrate specificities (e.g.,[1–5]). A number of companies (DuPont, Lonza, Dow, Diversa, BASF, DSM) have investigated the use of enzymatic nitrile hydrolysis in their processes. For instance, (R)-mandelic and (R)-chloromandelic acid have already been produced commercially by Mitsubishi Rayon Co. [5].

Nitrilases were discovered in the 1960s, when enzymes from barley [6] and \textit{Pseudomonas} [7] were purified. Since then, a large number of various nitrilases have been purified and characterized in bacteria, but only a few in eukaryotic organisms (plants and filamentous fungi; for a review see [3]). Nevertheless, the broad occurrence of nitrilases in filamentous fungi has been indicated by screening collection strains [8] and by gene and protein database searches [9].

We have recently been focusing our research on filamentous fungi as a source of new nitrilases with potentially different catalytic properties from those of the bacterial enzymes, and purified and characterized the nitrilases from \textit{Aspergillus niger} K10 [10] and \textit{Fusarium solani} O1 [11]. The latter enzyme exhibited similar specific activities to the nitrilase from \textit{Fusarium oxysporum} f. sp. melonis [12]. On the other hand, the nitrilase from the \textit{F. solani} IMI196840 strain described in one of the pioneering works on nitrilases in 1977 [13] exhibited a specific activity that was 87-times lower. This enzyme also differed from typical nitrilases in its subunit molecular mass (76 kDa [13] vs. 37–40 kDa in other fungal enzymes [10–12] and 38–46 kDa in most bacterial enzymes [3,4]).

The nitrilase in \textit{F. solani} IMI196840 was induced by benzonitrile as the sole source of C and N but the activity yield was very low [13,14]. Previously, we identified 2-cyanopyridine as a powerful nitrilase inducer, which was efficient in all the nitrilase-producing fungal strains examined (\textit{Aspergillus, Fusarium, Penicillium}) [15]. In this work, we used this compound to induce a high nitrilase activity in the \textit{F. solani} IMI196840 strain. The nitrilase purified and characterized by us from this strain is substantially different from that described in the same strain previously [13] but similar to the other two nitrilases described in \textit{Fusarium} genus [11,12], though some differences between these enzymes could be observed in terms of
their substrate specificity, pH and temperature profiles and stability.

2. Materials and methods

2.1. Chemicals

Substrates and authentic standards of the reaction products were purchased from Alfa Aesar (Germany), Sigma–Aldrich (USA) or Merck (Germany). Chemicals for protein sequencing were purchased from Applied Biosystems Inc. (USA).

2.2. Microorganisms and cultivation

Fusarium solani IMI196840 was purchased from the CABI BIOSCIENCE Genetic Resource Collection (Egham, Surrey, UK). The strain was grown in a two-stage culture. In the first stage, the fungus was grown in shaken 500 ml Erlenmeyer flasks with 200 ml of a medium consisting of (g l−1) sucrose 30, malt extract 5, yeast extract 5, NaN3O2 2 (pH 7.3 before sterilization). After a 48-h cultivation at 28°C the mycelium was harvested, washed with a modified Czapé-Dox medium (in g l−1): KH2PO4 1.0, MgSO4·7H2O 0.5, KCl 0.5, FeSO4·7H2O 0.001, CaCl2·6H2O 0.001, ZnSO4·7H2O 0.0067, yeast extract 0.1, pH 7.3 before sterilization) and resuspended in the same medium (in 200 ml/500 ml Erlenmeyer flasks). After incubating the suspension in shaken flasks for 1 h at 28°C, 2-cyanopyridine was added to a final concentration of 3 g l−1 and the incubation continued for a further 24 h. Afterwards the mycelium was harvested, washed with Tris/HCl buffer (50 mM, pH 8.0), frozen at −80°C, lyophilized overnight and stored at −20°C.

Fusarium solani O1 deposited in the Culture Collection of Fungi, Charles University, Prague (under accession number CCF 3635), was cultivated as described previously [11].

2.3. Preparation of cell-free extract

The enzyme was purified from a cell-free extract, which was obtained by grinding the lyophilized mycelium with a pestle and mortar. The homogenate was resuspended in 50 mM Tris/HCl buffer (pH 8.0) containing 0.8 M (NH4)2SO4 and sonicated in an ultrasonic bath (2 × 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 (10000 × g, 4°C, 30 min).

2.4. Enzyme purification

The cell-free extract was diluted twice with 50 mM Tris/HCl buffer (pH 8.0) containing 0.8 M (NH4)2SO4, and sonicated in an ultrasonic bath (2 × 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 (10000 × g, 4°C, 30 min).

2.5. Analytical size exclusion chromatography

A TSK G3000SW column (Toosh Bioscience, Germany) was used to determine the molecular mass of the native protein. The column was equilibrated with 50 mM Na/K phosphate buffer, pH 7.0, 150 mM NaCl and the flow rate was 1.5 ml min−1. A gel filtration HMW calibration kit (GE Healthcare, UK) for the range 158–669 kDa was used for calibration.

2.6. Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to Laemmli [16] in 12% polyacrylamide slab gels with protein molecular weight standards in the range of 14.4–97 kDa (GE Healthcare, UK).

2.7. Protein assay

The amount of protein was determined according to Bradford [17] using bovine serum albumin as the standard.

2.8. Mass spectrometry analysis

The protein band of the purified enzyme was manually excised from the SDS polyacrylamide gel. After destaining and washing (acetone/absolute alcohol), the digestion with trypsin (50 µg ml−1) was performed in 50 mM 4-ethylmorpholine acetate buffer (pH 8.1) overnight at 37°C. The tryptic peptides were extracted and analyzed after desalting with a UltraFlex III MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics) and identified based on peptides UPT spectra using the MS/MS ion search of Mascot program (Matrix Science) or de novo sequencing. Homologous proteins were searched for in the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.9. CD and fluorescence spectroscopy

Circular dichroism spectra were recorded at room temperature (22°C) using a Jasco-J-810 spectropolarimeter (Jasco, Japan). Data were collected from 185 to 260 nm, at 100 nm min−1, 1 s response time and 2 nm bandwidth using a 0.1 cm quartz cuvette containing the enzyme in 50 mM potassium phosphate buffer (pH 7.5). Each spectrum was the average of ten individual scans and was corrected for absorbance caused by the buffer. Collected CD data were expressed in terms of the mean residue ellipticity (θ220 nm) using the equation:

\[
\theta_{220}\text{nm} = \frac{1000 \times A_{220}}{c \times l} \text{deg cm}^2 \text{dmol}^{-1}
\]

where θ220 nm is the observed ellipticity in degrees, M is the protein molecular weight, n is the number of residues, 1 is the cell path length, c is the protein concentration (0.22 and 0.25 mg ml−1 for nitrilases from F. solani O1 and F. solani IMI196840, respectively) and the factor 100 originates from the conversion of the molecular weight to mg dmol−1. Secondary structure content was evaluated from the spectra using CD Spectra Deconvolution program [18] and SEL Consistent method [19] implemented in the program DICHROWEB [20,21]. Thermal unfolding of the enzymes was followed by monitoring the ellipticity at 221 nm over the temperature range of 20–80°C, with a resolution of 0.1°C, at a heating rate of 1°C min−1. Recorded thermal denaturation curves were roughly normalized to represent signal changes between approximately 1 and 0 and fitted to sigmoidal curves using software Origin 6.1 (OriginLab, Massachusetts, USA). The melting temperatures (Tm) were evaluated as the midpoint of the normalized thermal transition.

Fluorescence spectra were acquired by using spectrofluorimeter Fluorolog-Max-4F (HORIBA Jobin Yvon, USA) at room temperature (22°C) from 285 to 450 nm. Fluorescence emission spectra were measured with an excitation bandwidth of 1 nm, an emission bandwidth of 1 nm; and scan speed of 50 nm min−1 using a 0.5 cm quartz cuvette. The excitation wavelength was 280 nm. The sample concentrations were used as used for CD measurements. Each spectrum was corrected for fluorescence caused by the buffer.

2.10. Nitrilase assays

2.10.1. Spectrometric method

A rapid, semiquantitative detection of the nitrilase activity in fractions obtained by purification steps was performed by monitoring the absorption of benzoic acid at 238 nm (ε = 3300 M−1 cm−1) with a Saphire® plate reader (Tecan, USA). The reaction proceeded on UV-transparent 96-well plates (Nunc, USA) containing 15 µl of 10 mM benzylamine in methanol, 250 µl Tris/HCl (50 mM, pH 8.0) and 5 µl of the enzyme solution at 45°C. The reaction was quenched after 5 min by the addition of 30 µl of 1 M HCl. A reaction mixture with the same amount of HCl added at zero reaction time was used as the blank.

2.10.2. HPLC method

The nitrilase activity was assayed with 25 mM benzonitride (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 activity of the enzyme in the presence of inhibitors and cosolvents was also assayed under these conditions in reaction mixtures containing various metal ions, cosolvents or other additives.

The substrate specificity was assayed with a 25 mM concentration of various nitriles under the same conditions except for bromoxynil and isoxynil, which were examined at 0.5 mM concentrations (due to their low solubility) and prolonged reaction times (30 min), after which methanol (0.1 ml/0.1 ml of the reaction mixture) was added instead of HCl to stop the reaction, while avoiding substrate precipitation. Optimum pH was determined using 50 mM Britton–Robinson (acetate/boric acid/phosphoric acid/NaOH) buffers (pH 4–12) at 45°C. Optimum temperature was determined for reactions performed at pH 8.0 (50 mM Tris/HCl buffer) and various temperatures (25–60°C). The concentrations of benzonitrile, its analogues and the corresponding reaction products (acids, amides) were analyzed using a Chromolith Flash RP-18 (Merck; 25 mm × 4.6 mm) in a mobile phase consisting of acetonitrile (10–25%, v/v) in water and 0.1% (v/v) H3PO4 at a flow rate of 2 ml min−1 and 35°C. Heterocyclic nitriles and their products were analyzed as described previously [22]. Results of enzymatic assays were calculated from four independent measurements.
3. Results and discussion

3.1. Nitrilase induction and purification

The mycelium of *F. solani* IMI196840 produced high levels of nitrilase activity (up to 1500 U of nitrilase l\(^{-1}\)) when pre-grown in a rich medium and then transferred to a mineral medium with 2-cyanopyridine as nitrilase inducer. This activity was about 380-times higher than that previously reported in the same strain. In that work, a cell-free extract containing only about 59 U was obtained from 15 l of culture. Moreover, the specific activity of the cell-free extract (approx. 7 U mg\(^{-1}\) protein) was also much higher than in the previous work (0.088 U mg\(^{-1}\) protein [13]).

A striking difference was also observed between the specific activities of the purified enzymes. The new nitrilase, which was purified to near homogeneity (Fig. 1) with an about 20-fold increase in specific activity and 27% yield (Table 1), exhibited an activity of approximately 144 U mg\(^{-1}\) protein for benzonitrile, while that of the previous enzyme was significantly lower (1.66 U mg\(^{-1}\) protein for the same substrate). Hence, different enzymes were probably induced in this strain under different cultivation conditions. On the other hand, the specific activity of the new enzyme was similar to those of nitrilases purified from *F. solani* O1 (156 U mg\(^{-1}\) protein) [11] and *F. oxysporum* f. sp. *melonis* (143 U mg\(^{-1}\) protein) [12].

![Fig. 1. SDS-PAGE of nitrilase samples stained with Cooomassie Brilliant Blue R-250.](Image)

Lane 1, molecular weight marker; lane 2, after Q-Sepharose, lane 3, after Sephacryl S-200; lane 4, after Phenyl Sepharose; lane 5, cell-free extract.

(Fig. 1). This subunit size was the same or similar to other nitrilases from the *Fusarium* genus (Table 2).

The corresponding band in the electrophoreogram of the purified protein was identified as a putative nitrilase by mass spectrometric analysis of tryptic fragments (Fig. 2). Similar sequences were found in the CN hydrolase superfamily, primarily in hypothetical proteins from *Aspergillus oryzae* RIB40 (XP_001824866), *Gibberella zeae* PH-1 (XP_386656.1), *Gibberella moniliformis* (AFB83489.1) and *Nectria haematococca* mpVI 77-13-4 (EEU37388). Previous sequencing of tryptic and Asp-N digests of the nitrilase from *F. solani* O1 suggested its similarity to the same proteins (Fig. 2). No amino acid sequence information has been available for the other two nitrilases from *Fusarium*.

Most nitrilases form homooligomers of high molecular mass and the enzyme from strain IMI196840 with a molecular mass of 550 kDa as determined by gel filtration was typical in this respect (Table 2). The previous enzyme from the same strain also exhibited a similar molecular weight (620 kDa), though the size of its subunit was different [13].

The enzyme co-purified with a protein of about 60-kDa size, probably a chaperone. Similar phenomena were observed during

### Table 1

Purification of nitrilase from *Fusarium solani* IMI196840.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein [mg]</th>
<th>Specific activity [U mg(^{-1})]</th>
<th>Total activity [U]</th>
<th>Yield [%]</th>
<th>Purification [fold]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>67.6</td>
<td>7.1</td>
<td>483</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Hydrophobic chromatography on Phenyl Sepharose</td>
<td>9.7</td>
<td>39.2</td>
<td>379</td>
<td>78.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Gel filtration on Sephacryl S-200</td>
<td>3.8</td>
<td>64.0</td>
<td>243</td>
<td>50.3</td>
<td>9.0</td>
</tr>
<tr>
<td>Anion exchange on Q-Sepharose</td>
<td>0.9</td>
<td>144.0</td>
<td>130</td>
<td>26.9</td>
<td>20.3</td>
</tr>
</tbody>
</table>

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

### Table 2

Comparison of structural properties of nitrilases in *Fusarium* genus.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Molecular mass (kDa)</th>
<th>Secondary structures (%)</th>
<th>Melting temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subunit</td>
<td>Holoenzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. solani</em> IMI196840</td>
<td>40</td>
<td>550</td>
<td>α-Helix 30.0, β-sheet 21.0, turn 16.4, others 32.6</td>
<td>49.3</td>
</tr>
<tr>
<td><em>F. solani</em> O1</td>
<td>76</td>
<td>620</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>melonis</em></td>
<td>40</td>
<td>580</td>
<td>α-Helix 30.0, β-sheet 20.8, turn 16.4, others 32.8</td>
<td>47.8</td>
</tr>
</tbody>
</table>

n.a. = not available.

* Different-sized oligomers produced by non-denaturing electrophoresis
the purification of the nitrilase from A. niger K10 [10] and bacterial nitrilases [23,24].

Far-UV CD spectra (Fig. 3A) of nitrilase enzymes from F. solani IMI196840 and F. solani O1 showed one positive peak at 195 nm and two negative features at about 221 and 208 nm characteristic of $\alpha$-helical content [25]. Both enzymes exhibited similar intensity of the CD spectra, suggesting nearly identical content of their secondary structure. Protein secondary structure prediction based on measured CD data is summarized in Table 2. At the same time, thermally induced denaturation of both nitrilases was tested. Determined melting temperatures indicated slightly higher thermal stability of nitrilase from F. solani IMI196840 ($T_m = 49.3^\circ C$) compared to that from F. solani O1 ($T_m = 47.8^\circ C$). Both enzymes exhibited also the same shape and intensity of fluorescence spectra (Fig. 3B), indicating that their overall tertiary structures were almost identical.

3.3. Effect of temperature and pH

The enzyme exhibited maximal activity at 45 °C. Hence, it was similar to the other three nitrilases in genus Fusarium, which exhibited their temperature optima between 40 °C and 50 °C (Table 3). The new enzyme was more active at higher temperatures than the nitrilase from F. solani O1 (Fig. 4A). This observation was in accordance with the lower melting temperature of this latter enzyme (see above).

![Fig. 2](image-url) **Fig. 2.** Fragments of nitrilases from *Fusarium solani* IMI196840 and *Fusarium solani* O1 [11] and their similarity with the corresponding amino acid sequences translated from genes for hypothetical CN hydrolases in *Aspergillus oryzae* RIB40 (XP_001824866), *Gibberella moniliformis* (ABF83489.1), *Gibberella zeae* PH-1 (XP_386656.1) and *Nectria haematococca* mpVI 77-13-4 (EU373488). Mismatching amino acids are highlighted.

![Fig. 3](image-url) **Fig. 3.** Far-UV circular dichroism spectra (A) and fluorescence spectra (B) of nitrilases from *Fusarium solani* IMI196840 (squares; dashed line) and *Fusarium solani* O1 (circles; continuous line). CPS = counts per second.

All three enzymes from *F. solani* had similar pH optima around pH 8 (Table 3). The new enzyme exhibited a narrower pH optimum than that from *F. solani* O1 (Fig. 4B). The broadest pH optimum (6–11) was determined for the enzyme from *F. oxysporum* f. sp. *melonis* [12].

### 3.4. Substrate specificity and chemoselectivity

The new enzyme showed high specific activities towards some (hetero)aromatic nitriles (Table 4), as do other fungal nitrilases [10–13]. It hydrolyzed 4-cyanopyridine (its best substrate) at a high specific rate of 312 U mg\(^{-1}\) protein. The nitrilase from *A. niger* exhibited a higher activity for 4-cyanopyridine (411 U mg\(^{-1}\) protein) but it formed a high amount of the side product isonicotinamide (about one-third of the total product) [10]. On the other hand, the new enzyme produced low amounts of amides from all the substrates examined (<2% of total product) as do the enzymes from *F. solani* O1 [11] and *F. oxysporum* f. sp. *melonis* [12] (Table 3). 3-Cyanopyridine was hydrolyzed at a lower rate (59 U mg\(^{-1}\) protein), which, however, was still higher than in other fungal or bacterial nitrilases [9].

Compared to nitrilases previously characterized in the same strain and in *F. solani* O1, the new enzyme transformed benzonitrile analogues at lower relative rates (Table 4). In this respect it rather resembled the nitrilase from *F. oxysporum* f. sp. *melonis*.

**Table 3**

Comparison of catalytical properties of nitrilases in *Fusarium* genus.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Specific activity (U mg(^{-1}) protein)(^a)</th>
<th>Amide (% in total product)</th>
<th>T optimum (°C)</th>
<th>pH optimum</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. solani</em> IMI196840</td>
<td>144</td>
<td>≤2</td>
<td>45</td>
<td>8</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>1.66</td>
<td>n.a.</td>
<td>50</td>
<td>7.8–9.1</td>
<td>[13]</td>
</tr>
<tr>
<td><em>F. solani</em> O1</td>
<td>156</td>
<td>≤3</td>
<td>40–45</td>
<td>7–9</td>
<td>[11]</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>melonis</em></td>
<td>143</td>
<td>4–6</td>
<td>40</td>
<td>6–11</td>
<td>[12]</td>
</tr>
</tbody>
</table>

\(^a\) n.a. = not available.

\(^{1}\) Determined with benzonitrile as substrate.

**Table 4**

Substrate specificity of purified nitrilase from *F. solani* IMI196840.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity ± SD [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzonitrile</td>
<td>100</td>
</tr>
<tr>
<td>4-Cyanopyridine</td>
<td>217 ± 9</td>
</tr>
<tr>
<td>3-Cyanopyridine</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>2-Cyanopyridine</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>3-Tolunitrile</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>4-Tolunitrile</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>3-Chlorobenzonitrile</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>4-Chlorobenzonitrile</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>3-Hydroxybenzonitrile</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>3-Aminobenzonitrile</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

Nitrilase activity was assayed with 25 mM substrates (see Section 2 for details). The specific activity for benzonitrile (144 U mg\(^{-1}\)) was taken as 100%. The strong inhibitory effect of Ag\(^{+}\), Hg\(^{2+}\) was generally observed in nitrilases; this was predictable as they are enzymes containing a catalytically active cysteine (e.g., [10–13,26]). In comparison with the nitrilase from *F. solani* O1 [11], the new enzyme was more inhibited by Cr\(^{3+}\) but less by Cu\(^{2+}\) and Fe\(^{3+}\). It is not yet clear to what extent these and other differences between the two enzymes (see above) were caused by the differing purification states of the enzymes, the new enzyme being co-purified with a considerable amount of a contaminating protein, probably a chaperone.

**Fig. 4.** Effect of temperature (A) and pH (B) on the relative activity of nitrilases from *Fusarium solani* IMI196840 (squares) and *Fusarium solani* O1 (diamonds). The specific activities of the enzymes at 45 °C and pH 8.0 (144 and 156 U mg\(^{-1}\), respectively) were taken as 100%. Nitrilase activity was assayed with 25 mM substrates (see Section 2 for details).
Nitrilase activity was assayed with 25 mM benzonitrile (see Section 2 for details). Appreciable activity was also found for 3-cyanopyridine nitrile, 4-cyanopyridine, which can serve as a precursor of tuberculostatics. Furthermore, the enzyme has much more potential as a biocatalyst (first of all the smaller subunit size and higher specific activity of the new enzyme) strongly suggested that different enzymes were induced (by the inducer). In comparison to the previous results reported in [20,21], we increased the nitrilase activity in this strain 380-fold with 2-cyanopyridine as inducer. In accordance with its higher melting temperature, an even higher thermostability and higher resistance to organic solvents. Hence, the enzyme has much more potential as a biocatalyst.

### 4. Conclusion

Using *F. solani* IM196840, a nitrilase-producing isolate from a bromoxynil-treated field in Northern Ireland, we increased the nitrilase activity in this strain 380-fold with 2-cyanopyridine as the inducer. In comparison to the previous results reported in 1977 [13], the fungus produced a different nitrilase with an 87-fold higher specific activity for benzonitrile and with a different subunit molecular weight (40 vs. 76 kDa). A mycelium with high nitrilase activity could be easily prepared in appreciable quantities. Hence, the enzyme has much more potential as a biocatalyst than that reported previously in the same strain. Comparison of the two nitrilases in terms of their structural and catalytic properties (first of all the smaller subunit size and higher specific activity of the new one) strongly suggested that different enzymes were induced using the different cultivation protocols. The highest specific activity of the new enzyme was found for an industrially important nitrile, 4-cyanopyridine, which can serve as a precursor of tuberculostatics. Appréciable activity was also found for 3-cyanopyridine (precursor of nicotinic acid – vitamin B3). In terms of their specific activities towards these substrates, the fungal nitrilases (from the *Fusarium* and *Aspergillus* genera) seem to surpass most of the nitrilases from bacteria. The enzymes from *Fusarium* genus have been shown to be superior, as far as their thermostability and selectivity (product purity) are concerned. Compared to the other nitrilase from the same species (*F. solani* O1), the new enzyme showed, in accordance with its higher melting temperature, an even higher thermostability and higher resistance to organic solvents.

## Acknowledgements

Financial support through projects IAA500200708 (Grant Agency of the Academy of Sciences of the Czech Republic), LC06010, OC09046 (Ministry of Education of the Czech Republic), 305/09/H008 (Czech Science Foundation), FT-TA/043 (Ministry of Industry and Trade of the Czech Republic) and the institutional research concept AV0Z50200510 (Institute of Microbiology) is gratefully acknowledged. The authors also wish to thank Prof. J. Damborsky (Loschmidt Laboratories, Masaryk Univ. Brno, CZ) for providing the facilities for CD and fluorescence spectra measurement.

## References

14. Please note that the total activity of nitrilase in strain *F. solani* IM196840 was cited incorrectly in our previous article (Ref. [9]). The correct value is 3.9 U L−1.

<table>
<thead>
<tr>
<th>Additive Activity [%] at final concentration of additive ± SD</th>
<th>1 mM</th>
<th>5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>AgNO₃</td>
<td>1.7 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Al₂(SO₄)₃</td>
<td>0.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>CoCl₂</td>
<td>75.8 ± 4</td>
<td></td>
</tr>
<tr>
<td>CuSO₄</td>
<td>88.3 ± 2</td>
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</tr>
<tr>
<td>Cr₂O₃</td>
<td>27.8 ± 8</td>
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</tr>
<tr>
<td>FeCl₃</td>
<td>43.7 ± 4</td>
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</tr>
<tr>
<td>HgCl₂</td>
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<tr>
<td>MgSO₄</td>
<td>85.3 ± 2</td>
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<tr>
<td>MnSO₄</td>
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<tr>
<td>NiSO₄</td>
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<tr>
<td>(CH₃CO₂)₂Pb</td>
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<tr>
<td>ZnSO₄</td>
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<tr>
<td>EDTA</td>
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<tr>
<td>Dithiothreitol</td>
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<tr>
<td>Mercaptopropenol</td>
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</tr>
</tbody>
</table>

SD = standard deviation.