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Journal of Molecular Catalysis B: Enzymatic 29 (2004) 227–232
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Nitrile biotransformation by *Aspergillus niger*

Radka Šnajdrová[a], Veronika Kristová-Mylerová[a], Dominique Crestia[b], Konstantina Nikolaou[a], Marek Kuzma[a], Marielle Lemaire[b], Estelle Gallienne[b], Jean Bolte[b], Karel Bezouška[c], Vladimír Křen[a], Ludmila Martínková[a,*]

[a] *Institute of Microbiology, Laboratory of Biotransformation, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic*
[b] *University of Blaise Pascal, SEESIB, 24 Av. des Landais, F-63 177 Aubière Cedex, France*
[c] *Department of Biochemistry, Faculty of Science, Charles University Prague, Hlavova 8, CZ-128 40 Prague, Czech Republic*

Received 31 July 2003; received in revised form 24 November 2003; accepted 1 December 2003

Available online 9 April 2004

Abstract

A nitrile-converting enzyme activity was induced in *Aspergillus niger* K10 by 3-cyanopyridine. The whole cell biocatalyst was active at pH 3–11 and hydrolyzed the cyano group into acid and/or amide functions in benzonitrile as well as in its *meta*- and *para*-substituted derivatives, cyanopyridines, 2-phenylacetonitrile and thiophen-2-acetonitrile. Amides constituted a significant part of the total biotransformation products of 2- and 4-cyanopyridine, 4-chlorobenzonitrile, 4-tolunitrile and 1,4-dicyanobenzene, while α-substituted acrylonitriles gave amides as the sole products.

Keywords: *Aspergillus niger*; Nitrile-converting enzymes; Nitrile hydratase; Nitrilase; Amidase

1. Introduction

In comparison to nitrile-converting enzymes in bacteria, few data are available about nitrile metabolism in fungi though the first studies on this topic were published as early as in the 1960s. It was shown that indolylacetonitrile was hydrolyzed by fungi belonging to *Fusarium*, *Giberella*, *Aspergillus* and *Penicillium* [1,2] and 2-aminopropionitrile [3] and 4-amino-4-cyanobutyric acid [4] by *Basidiomyces*. Later on, the ability to hydrolyze aliphatic di- and trinitriles was reported in *Fusarium merismoides* [5,6] and *Fusarium solani* [6] and utilization of 2-hydroxynitriles in *Torulopsis candida* [7]. Formation of an intermediate amide was not reported for nitrile conversions catalyzed by any of the above fungi. This suggested that these strains hydrolyzed nitriles via the nitrilase pathway. In the recent years, nitrile-hydrolyzing strains of *Candida famata* [8], *Candida guilliermondi* [9,10], *Cryptococcus* sp. [11], *Rhodotorula glutinis* [11] and of other yeasts [11–13] were reported. In yeasts, nitriles are probably hydrolyzed via the nitrile hydratase/amidase pathway as corresponding amides were also accepted as substrates [8–11]. In addition, amides were detected as products besides the carboxylic acids [9,11]. According to our knowledge, few fungal nitrile-converting enzymes have been purified and characterized, namely nitrilases from *Fusarium solani* [14] and *Fusarium oxysporum* f. sp. *melonis* [15] and a nitrile hydratase from *Myrothecium verrucaria* [16]. Enzymes that hydrate HCN into formamide are probably more common in fungi than those acting on organic cyanides. Cyanide hydratases have been reported in *Fusarium lateritium* [17], *Fusarium solani* [18], *Leptosphaeria maculans* [19] and *Gloeocercospora sorghi* [20]. However, the cyanide hydratase from *F. lateritium* showed also a low but significant nitrilase activity towards benzonitrile, propionitrile and acetonitrile [21].

We performed screening for organic nitrile-hydrolyzing microorganisms among about 100 strains of filamentous fungi from culture collections and found thirteen 3-cyanopyridine-utilizing cultures belonging to *Aspergillus*, *Fusarium*, *Penicillium* and *Talaromyces* (unpublished results). The present work describes the nitrile-converting ability of one of these microorganisms, *Aspergillus niger*

* Corresponding author. Tel.: +420-296-442-569; fax: +420-296-442-509.
E-mail address: martinko@biomed.cas.cz (L. Martínková).

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doi:10.1016/j.molcatb.2003.12.012

K10, that is, to the best of our knowledge, the first example of an *Aspergillus* strain with a broad specificity towards (hetero)aromatic and (aryl)aliphatic nitriles.

2. Experimental

Chemicals: Authentic standards of cyanobenzamides **6c** and **7c** were prepared as described previously [22]. Cyanobenzoic acids **6b** and **7b** were kindly supplied by the Department of Organic Chemistry of TU Graz, Austria.

α-substituted acrylonitriles **12a** and **13a** were prepared by the Baylis–Hillman reaction between acrylonitrile and acetaldehyde or benzaldehyde, respectively, in the presence of diazabicyclooctane (DABCO) [23,24]. Compound **14a** was prepared by the Barbier type reaction as described previously for 2-(2-hydroxy-3,3-dimethoxypropyl)acrylonitrile [25], while acetaldehyde was used instead of 2,2-dimethoxyacetaldehyde. Authentic standards of α-substituted acrylamides **12c** and **13c** were prepared using whole cells of *Rhodococcus equi* A4 (deposited in the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic) [26]. Compound **12a** (25 mM) was hydrated by the cell suspension ($A_{610} = 2.5$) in a phosphate buffer (54 mM, pH 7.5; containing 5% (v/v) methanol as a cosolvent) within 1 h at 30 °C with shaking. Compound **13a** (25 mM) was hydrated by the cell suspension ($A_{610} = 20$) within 2.5 h under the same conditions. For hydration of compound **14a** the purified nitrile hydratase [27] from *Rhodococcus equi* A4 was used, as whole cells gave a mixture of acid **14b** and amide **14c**. The enzyme (15 μg ml^{-1} of the reaction mixture) was applied for hydration of **14a** (10 mM) at the same conditions as the whole cells and the reaction was complete after 3 h. The reaction mixtures (after removal of whole cells if necessary) were lyophilized and extracted with dry methanol to afford amides **12c**, **13c** and **14c**.[1]

All other substrates and authentic standards of biotransformation products were of analytical-grade purity and supplied from standard commercial sources.

Microorganism and cultivation: *A. niger* K10 (deposited in the Culture Collection of Fungi, Charles University, Prague, Czech Republic) was grown in 300 ml baffled Erlenmeyer flasks containing a modified Czapek-Dox medium (sucrose 30 g/l, $NaNO_3$ 2 g/l, K_2HPO_4 1 g/l, $MgSO_4{\cdot}7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4{\cdot}7H_2O$ 0.01 g/l, $CoCl_2{\cdot}6H_2O$ 0.001 g/l, $ZnSO_4{\cdot}7H_2O$ 0.0067 g/l) supplemented with 3-cyanopyridine (2 g/l) or both 3-cyanopyridine and $NaNO_3$ (2 g/l each) as nitrogen sources. The cultivation proceeded on an orbital shaker at 200 rpm and 28 °C for 3 days.

Biotransformation using whole cells: The mycelium from 25 ml of cultivation medium (average dry cell weight of 30 mg corresponding to 550 mg of wet cell weight) was filtered off, washed with sodium/potassium phosphate buffer (50 mM, pH 7) and resuspended in 10 ml of the same buffer with 2–10 mM of the substrate. Methanol (2%, v/v) was added to the reaction mixtures except for those containing 2-, 3- or 4-cyanopyridine. The reactions were carried out in shaken flasks on an orbital shaker at 200 rpm and 28 °C. For determination of pH optimum, the same amount of the mycelium was thoroughly washed with distilled water and the reaction mixtures were made of 50 mM buffers (phenylacetic acid/boric acid/phosphoric acid/NaOH buffer for pH 2 and 11–12, Na phosphate/citrate for pH 4–5, Na/K phosphate for pH 6–7, Tris/HCl for pH 8–9 and glycin/NaOH buffer for pH 10), 10 mM of benzonitrile and 2% (v/v) of methanol.

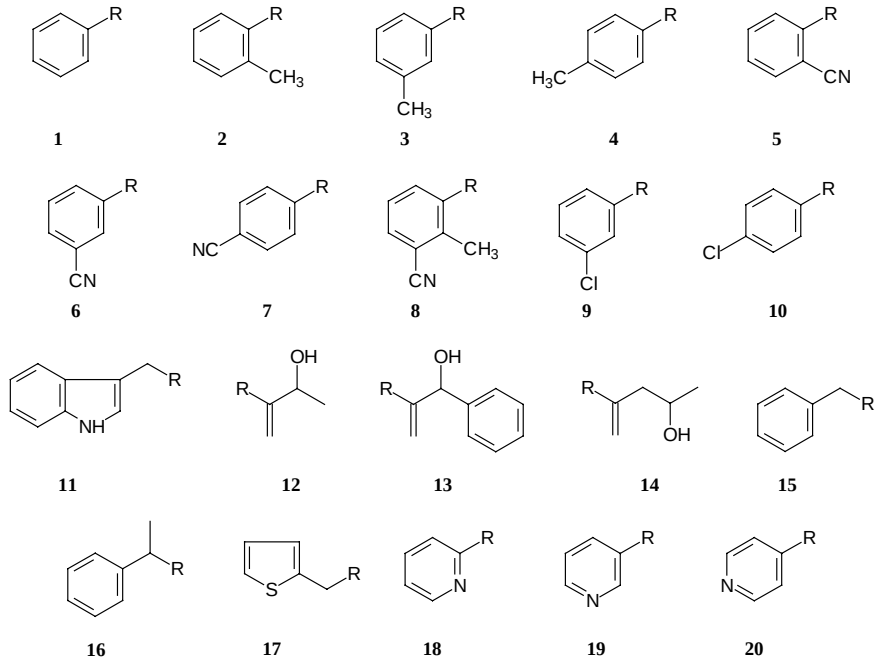
Biotransformation using cell extracts: The mycelium was filtered off, washed with distilled water and ground to powder in a mortar under liquid nitrogen. The mycelium powder was suspended in a Tris/HCl buffer (50 mM, pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol and disintegrated by ultrasonication in a Cole-Parmer (USA) 4710 sonifier (10 times 10 s at intervals of 50 s). The supernatants obtained by centrifugation at 13 000 × *g* for 30 min at 4 °C were used for enzyme assays. Hydrolysis of benzonitrile (2 mM) was carried out in different buffers (see biotransformations using whole cells) at 28 °C and with shaking (850 rpm, Thermomixer Compact Eppendorf).

Analytical methods: At intervals, the reactions catalyzed by whole cells were quenched with 1 M HCl (0.1 ml per 1 ml of sample) and, if necessary, methanol (1 ml per 1 ml of sample) was added to solubilize the substrate and/or products. The cells or the precipitated protein were removed by centrifugation. Using authentic standards (see Chemicals), the products of the biotransformations were identified by HPLC and by UV spectra recorded by the photodiode array detector 996 (Waters) at 210–280 nm. The HPLC system consisted of the solvent delivery system 600 and the PDA detector 996. Cyanopyridines **18a**–**20a** and the corresponding amides and acids (Scheme 1) were determined using an ACE column (5 μm, 4.0 mm × 250 mm, Advanced Chromatography Technologies) and a mobile phase consisting of 10% (v/v) acetonitrile and 0.1% (v/v) H_3PO_4. A Nova-Pak C_{18} column (5 μm, 3.9 mm × 150 mm, Waters) was employed for determination of nitriles **1a**–**11a**, **15a**–**17a** and the corresponding amides and/or carboxylic acids (Scheme 1) using a mobile phase containing 25% (v/v) acetonitrile and 0.1% (v/v) H_3PO_4. Substituted acrylonitriles **12a**–**14a** and the corresponding amides (Scheme 1) were analyzed using the same column and a mobile phase containing 10% (v/v) acetonitrile and 0.1% (v/v) H_3PO_4. Quantification was performed at local spectral maxima in the range from 210 to 270 nm or at 210 nm when no local maxima of the compounds were detected in this wavelength range.

3. Results and discussion

An intracellular benzonitrile-hydrolyzing activity of *A. niger* K10 was induced by 3-cyanopyridine, both in pres-

[1] NMR data available on request.



a R = CN

b R = COOH

c R = $CONH_2$

Scheme 1.

ence or absence of sodium nitrate. Mycelia grown in the absence of 3-cyanopyridine (on sodium nitrate as sole source of nitrogen) did not convert benzonitrile.

The benzonitrile-hydrolyzing cells were active at pH 3–11 and the reaction was the fastest at pH 7 (see Fig. 1). A typical ratio of the molar concentrations of biotransformation products—benzoic acid and benzamide—was 95:5. Only few biocatalysts were reported to be active at such a broad pH range, i.e. the nitrile hydratase from *Rhodococcus* sp. (between pH 3 and 10) [28] and *Rhodococcus* sp. polyvinyl alcohol-entrapped whole cells (between pH 2 and 13) [29]. *A. niger* K10 grows in pellets (of approximately 2 mm diameter) under the conditions applied. In the pellet cultures, the intracellular enzymes are probably protected from adverse exogeneous effects due to transport limitation. This hypothesis was supported by the observation that the nitrile-hydrolyzing enzyme in the cell extract was not active at pH $\leq$ 5 (see Fig. 1).

The biotransformation of most examined nitriles (Scheme 1) gave the corresponding carboxylic acids (Table 1). Benzonitrile (**1a**) was the best substrate of those examined herein. Benzoic acid was consumed by the fungus at later reaction stages. However, this pattern was valid only for pH 7 or less, while the metabolism of benzoic acid was impaired at pH 8–11. Therefore, further conversions, which employed higher concentrations of benzonitrile, were carried out at pH 8. In this way, 25 and 100 mM benzonitrile was transformed into benzoic acid by 90 and 76% within 24 and 48 h, respectively.

Similarly as benzoic acid, nicotinic acid produced by the hydrolysis of 3-cyanopyridine was also metabolized by the fungus at pH 7 but at a lower rate than benzoic acid. Steric hindrances were probably the cause of a lower reactivity of *meta*- and *para*-substituted benzonitriles (tolunitriles **3a** and **4a**, dicyanobenzenes **6a** and **7a** and chlorobenzonitriles **9a** and **10a**). Comparison of the smooth hydrolysis of 2-phenylacetonitrile (**15a**) and the negligible conversion of 2-phenylpropionitrile (**16a**) suggested a negative effect of the α-methyl substituent on the reaction rate. 3-Indolylacetonitrile (**11a**) as a bulky molecule was also a poor substrate. The *ortho*-substituents in 2-tolunitrile (**2a**), 1,2-dicyanobenzene (**5a**) and 2,6-dicyanotoluene (**8a**) hampered the hydrolysis totally.

The biocatalyst produced significant amounts of amides from some nitriles. Bioconversion of 1,4-dicyanobenzene (**7a**) afforded a mixture of 4-cyanobenzoic acid (**7b**) and 4-cyanobenzamide (**7c**; approximately 22% of the total products). No other products such as terephthalic acid or its mono or diamide were detected. The end product of the reaction was **7b**. Transformation of 4-chlorobenzonitrile (**10a**) and 4-tolunitrile (**4a**) also gave both the corresponding acids **4b** or **10b** and amides **4c** or **10c** (approximately 19 and 38% of the total products, respectively). On the other hand, in the product obtained from *meta*-substituted benzonitriles **3a**,

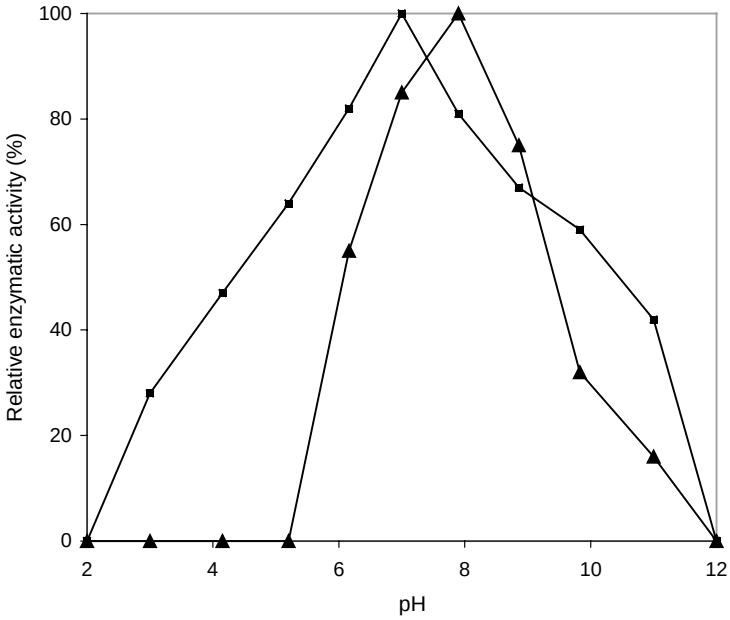


Fig. 1. Effect of pH on the benzonitrile-hydrolyzing activity of whole cells (■) and cell extract (▲) of *A. niger* K10. The reaction mixture contained 10 mM of benzonitrile, 2% (v/v) of methanol and the whole cells (approximately 3 mg of dry cell weight per ml^{-1}) or 2 mM of benzonitrile, 2% (v/v) of methanol and the cell extract (approximately 0.8 mg ml^{-1}) in different buffers (see Section 2). Samples were withdrawn after 2 h incubation at 28 °C and analyzed for benzonitrile, benzamide and benzoic acid by HPLC (see Section 2). Activity of whole cells at pH 7 (0.75 μmol of total products (benzamide and benzoic acid) h^{-1} mg^{-1} of dry cell weight) and activity of the cell extract at pH 8 (0.3 μmol of total products (benzamide and benzoic acid) h^{-1} mg^{-1} of protein) were taken as 100%. The pH drop was approximately 0.2 within 2 h of reaction in media with initial pH ≥ 8. No spontaneous formation of benzamide or benzoic acid was observed at pH ≤ 11.

6a and **9a** the corresponding amides **3c**, **6c** and **9c** gave less than 2% of the total product. Isonicotinamide (**20c**) was found as a product of the transformation of 4-cyanopyridine (**20a**; approximately 15% of the total products) in addition to isonicotinic acid (**20**). From 2-cyanopyridine (**18a**), the fungus gave picolinic acid (**18b**) and picolinamide (**18c**) in comparable amounts. Moreover, on conversion of substituted acrylonitriles **12a**–**14a** amides **12c**–**14c** were detected as the only products.

The formation of both amides and acids from nitriles corresponds with two alternative reaction patterns, involving either nitrile hydratase/amidase or nitrilase. Formation of amides as by-products or even the only products (in case of substituted acrylonitriles) may indicate presence of the former pathway. It may be concluded that the ratio of nitrile hydratase/amidase activities is higher for *para*-substituted benzonitriles than for the *meta*-substituted ones, the *para*-substitution imposing steric hindrances on the amidase. Nevertheless, detection of an amide is not an unambiguous evidence for the nitrile hydratase/amidase pathway as the production of amides from nitriles by nitrilases is known. Amides as by-products made up 4–6% of the total products in hydrolysis of nitriles by the nitrilase from *Fusarium oxysporum* f. sp. *melonis* [15]. Similarly, the nitrilase from *Rhodococcus* ATCC 39484 formed phenylacetamide (2% of the total product) from phenylacetonitrile [30]. *A. niger* K10, however, gave higher amide/acid ratios from some nitriles than these nitrilases. The formation of amides from nitriles was also pronounced with the nitrilase from *Arabidopsis thaliana* that converted 2-fluoroarylacetonitriles into 2-fluoroarylacetamides, while the corresponding acids formed only 15–30% of the total products [31]. Cyanide hydratase that shows a strong homology to nitrilases gives only the corresponding amide (formamide) from HCN [18]. Therefore, it is necessary to purify the nitrile-converting enzyme from *A. niger* K10 in order to assess to which type (nitrilase or nitrile hydratase) it belongs.

Benzamide (initial concentration 2 mM) was consumed by 80% by the biocatalyst (1.3 mg dry cell weight per ml^{-1}) within 24 h at pH 7, while benzoic acid was formed transiently. This finding suggested that the fungus produced an amidase. In fact, benzamidase activity was also reported in *Aspergillus nidulans* together with other amidases specific either for formamide (formamidase) or aliphatic amides (acetamidase) [32]. Nitrilase is another enzyme that is able to hydrolyze amides; this activity was described for the nitrilase from *Rhodococcus rhodochrous* [33]. Nevertheless, the specific activity of this enzyme towards benzamide was only 0.00022% of that towards benzonitrile. Thus, it is not probable that benzamide utilization was caused by nitrilase activity in *A. niger* K10.

Enzymes hydrating organic nitriles have been rarely reported in filamentous fungi. The nitrile hydratase from *Myrothecium verrucaria* showed a narrow substrate specificity towards cyanamide [16]; thus this enzyme and that of *A. niger* K10 clearly differ in substrate specificity. The

Table 1
Biotransformations of nitriles by whole cells of *A. niger* K10

Entry	Substrate (initial concentration, mM)	Reaction time (h)	Product	Yield (%)[a]
1	**1a** (10)	7	**1b**[b]	90
2	**1a** (25)	28	**1b**	90
3	**1a** (100)	48	**1b**	76
4	**3a** (10)	47	**3b**	45
5	**4a** (10)	47	**4b**	21
			4c	5
6	**6a** (4)	47	**6b**	30
7	**7a** (4)	16	**7b**	69
			7c	20
8	**9a** (10)	26	**9b**	68
9	**10a** (10)	26	**10b**	15
			10c	9
10	**11a** (2)	20	**11b**	5
11	**12a** (2)	26	**12c**	14
12	**13a** (2)	26	**13c**	29
13	**14a** (2)	26	**14c**	3
14	**15a** (10)	40	**15b**	27
15	**16a** (2)	22	**16b**	7
16	**17a** (10)	17	**17b**	54
17	**18a** (10)	35	**18b**	12
			18c	15
18	**19a** (10)	17	**19b**[c]	16
19	**20a** (10)	35	**20b**	85
20			**20c**	15

Substrates **1a**–**17a** (Scheme 1) were transformed in reaction mixtures (total volume 10 ml) containing Na/K phosphate buffer (50 mM, pH 7, for entries 1 and 4–20) or Tris/HCl buffer (50 mM, pH 8, for entries 2–3), whole cells of *A. niger* K10, and methanol (2%, v/v) as a cosolvent. Methanol was omitted for substrates **18a**–**20a**. The concentration of the biomass in conversions of **1a**–**16a** and **17a**–**20a** corresponded to 3.6 ± 0.3 and 2.0 ± 0.3 mg of dry cell weight ml^{-1}, respectively. Substrates/products were determined by HPLC (see Section 2 for details). Conversion of 2-tolunitrile **2a**, 1,2-dicyanobenzene **5a** and 2,6-dicyanotoluene **8a** by *A. niger* K10 was not detected.

[a] Determined by HPLC.

[b] Benzoic acid (**1b**) metabolized within 24 h.

[c] Nicotinic acid (**19b**) metabolized within 72 h.

substrate specificity of the *A. niger* enzyme was also different from that of the nitrilase from *Fusarium solani* [14] that showed no activity for 2-phenylacetonitrile. However, whole cells of *A. niger* K10 and the purified nitrilase from *Fusarium oxysporum* f. sp. *melonis* [15] showed some similar features in their substrate specificities (e.g., benzonitrile as the best substrate, lower activities for 4-tolunitrile and 2-phenylacetonitrile, no activity for 2-tolunitrile). The range of substrates accepted by the nitrile-hydrolyzing enzymes reported in the present work is narrower than that reported for some bacterial enzymes as *ortho*-substituted benzonitriles and bulky nitriles are not accepted. On the other hand, the broad pH range (3–11) for the whole cell fungal biocatalyst activity is a significant benefit. The potential of enzymatic nitrile hydrolysis that so far employed mainly bacterial enzymes may be extended by introducing enzymes from microorganisms distinct in evolution. The properties (stability, stereospecificity) of the nitrile-hydrolyzing enzyme from strain K10 will be therefore examined in future studies.

4. Conclusions

A. niger K10 transforms a broad variety of structurally distinct nitriles. The product of the hydrolysis of some substrates contains a high ratio of the corresponding amide or consists of the amide only. This pattern of nitrile conversion is novel for filamentous fungi that hydrolyze nitriles mostly via the nitrilase pathway. Thus, the nitrile-converting enzyme is either a nitrilase affording extremely large amounts of some amides or a nitrile hydratase. The latter enzyme possessing a broad substrate specificity has not yet been reported in a filamentous fungus. The broad pH optimum (3–11) of the fungal biocatalyst is infrequent for nitrile-hydrolyzing microorganisms. Utilization of the fungus at slightly acidic pH may be useful in biotransformations of nitriles unstable at neutral pH such as cyanohydrins.

Acknowledgements

Financial support of this work through COST D25/0002/02, the bilateral project Barrande 2002-020-1, the national grants A4020213 (GA AV ČR), ME 579 and OC D25.001 (MŠMT) and the institutional research concept MBÚ AVOZ 5020903 is gratefully acknowledged. We wish to thank Ms. M. Balvínová for her excellent technical assistance.

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