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ORIGINAL ARTICLE

Nitrile biotransformation by whole cells of *Aspergillus* sp. PTCC 5266

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Abstract

Aspergillus sp. PTCC 5266 exhibited nitrile-hydrating activity over a broad pH range from 6.0 to 10.0 at 26°C. It hydrated 4-nitrophenylacetonitrile, 2-chlorobenzonitrile and 3-chlorobenzonitrile to their corresponding carboxylic acids and amides, while benzyl cyanide, benzonitrile, 4-tolunitrile, cyclohexanecarbonitrile, 4-chlorobutyronitrile and isobutyronitrile gave carboxylic acids as the sole products. The maximum whole-cell nitrile-hydrating activity was observed at pH 7.0.

Keywords: *Aamide,* Aspergillus *sp. PTCC 5266, carboxylic acid, nitrile biotransformation, nitrile hydrating activity*

Introduction

Nitrile-hydrolyzing enzymes have found broad use in organic synthesis. Compared with nitrile-converting enzymes in bacteria, little information is available about nitrile-converting metabolism in fungi. In the majority of reports characterized nitrile hydratases/amidases and nitrilases have been of bacterial origin, with only two out of 21 well-characterized nitrilases being isolated from filamentous fungi (belonging to the genus *Fusarium*) (O'Reilly & Turner 2003).

There are two established metabolic pathways to convert nitriles to the corresponding carboxylic acids (Hann et al. 1999; Asano 2002; Martínková & Mylerová 2003): (1) nitrilase catalyzes the direct hydrolysis of nitriles to carboxylic acids ($RCN + 2H_2O \rightarrow RCOOH + NH_3$); and (2) nitrile hydratase (NHase) catalyzes the hydration of nitriles to amides ($RCN + H_2O \rightarrow RCONH_2$), which are subsequently hydrolyzed to carboxylic acid by the action of an amidase ($RCONH_2 + H_2O \rightarrow RCOOH + NH_3$) (Asano et al. 1980; Banerjee et al. 2002; Brandao et al. 2004).

Nitriles, in general, exhibit harmful effects on humans and the environment. Some of them (e.g. cyanohydrins, aliphatic saturated and unsaturated nitriles) are classified as toxic or even highly toxic compounds. Many of the nitrile-converting microbes and enzymes showed high specific activities and broad substrate specificities, which makes them promising for biotechnological applications. An important feature of most nitrile-converting enzymes is their activity with a broad range of nitriles, making them useful as biocatalysts for organic synthetic reactions, leading to valuable products and intermediates such as carboxylic acids or their amides (Martínková et al. 2009a) which have extensive applications in the synthesis of fine chemicals and pharmaceuticals. Thus, over the last 20 years, organonitrile biotransformation with resting cells and isolated enzymes has assumed major industrial importance (Wang et al. 2007). These reactions have been extensively studied and summarized in a number of reviews (Beard & Page 1998; Bunch 1998; Wang & Lin 2002; Cantarella et al. 2004; Wang 2005; Singh et al. 2006).

The aim of the present work was to investigate the ability of *Aspergillus* sp. PTCC 5266 in the biotransformation of aromatic and aliphatic nitriles (for structures, see Scheme 1).

Materials and methods

Materials

The nitriles used in the present study were purchased from Merck (Darmstadt, Germany). The culture media ingredients were from Scharlau (Barcelona, Spain). All nitriles and authentic standards, benzoic acid **5b** and benzamide **5c**, were purchased from

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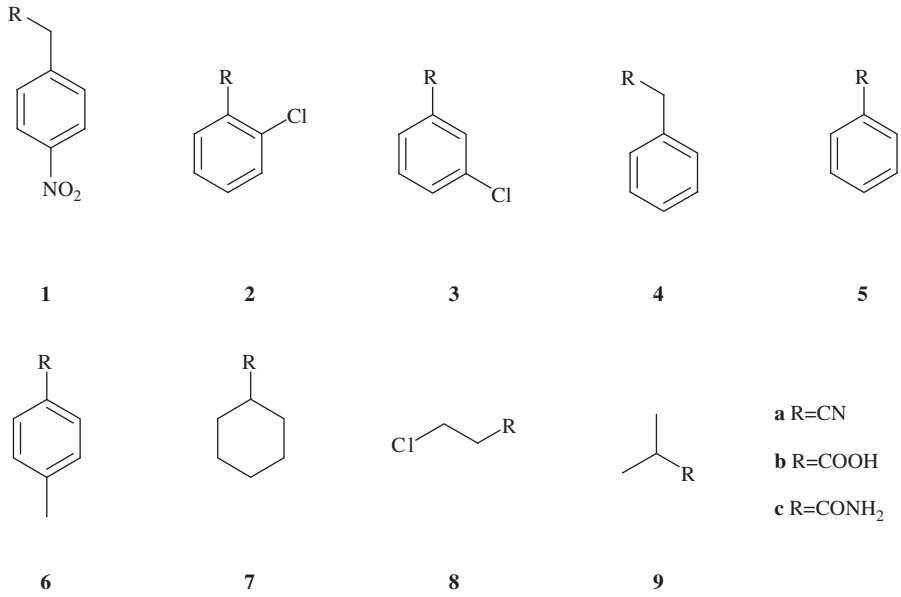

Scheme 1.

Merck. Authentic standards of other nitriles (corresponding carboxylic acids and amides) were prepared by nitrile hydrolysis. For preparation of carboxylic acids, the nitriles were heated under reflux with hydrochloric acid (6 M) for 24 h; for preparation of amides, the nitriles were heated under reflux with potassium hydroxide solution (2 M) for 1 h. Then the reaction mixture was extracted with chloroform and the structures of products were confirmed by their melting points and spectral data. All other chemicals were of analytical grade and procured from various commercial sources.

Microorganism and cultivation

The strain *Aspergillus* sp. PTCC 5266 (deposited in the Persian Type Culture Collection of Microorganisms, Iranian Research Organization for Science and Technology, Tehran, Iran) was employed in the present work. It was streaked on potato–glucose–agar plates (composition, g/L: glucose 20 g, diced potatoes 300 g, agar 15.0 g). All media were sterilized by autoclaving at 121°C for 20 min. A single colony was used to inoculate 100 mL of culture medium in a 250-mL Erlenmeyer flask. Culture medium was potato–glucose broth containing 20.0 g glucose and 300 g diced potatoes per liter of distilled water, adjusted to pH 7.0.

General procedure for biotransformation of nitriles using whole cells

The mycelium from 100 mL of cultivation medium (average dry cell weight of 300 mg, corresponding to 10 g wet cell weight) was filtered off after 48 h, washed with sodium phosphate buffer (50 mM, pH 7) and re-suspended in 100 mL of the same buffer with 10 or 50 mM of the substrate. The substrates were dissolved in dimethylsulfoxide (2%, v/v) and all of the substrate was added in one step. The reactions were carried out in shaking flasks on an orbital shaker at 120 rpm and 26°C. The reaction, monitored by TLC, was quenched after a period of time by removing the biomass through filtration. For determination of the pH optimum, the same amount of mycelium, thoroughly washed with distilled water, was added to the reaction mixtures made of 50 mM buffers (Na_2HPO_4/citrate for pH 5, Na_2HPO_4/NaH_2PO_4 for pH 6–7, Tris–HCl for pH 8–9 and glycine/NaOH buffer for pH 10), 10 mM of benzonitrile and 2% (v/v) of dimethylsulfoxide.

Enzyme assays

Hydrolyzing activities for benzonitrile and other nitriles (10 mM each) were compared in a system containing Na_2HPO_4/NaH_2PO_4 buffer (50 mM, pH 7), *Aspergillus* sp. PTCC 5266 cells (1 mg dry cell weight) and dimethylsulfoxide (2%, v/v) as co-solvent (total volume of 10 mL) for all substrates. The reactions were carried out at 26 °C for 1 h. The reactions were stopped by adding 0.05 mL of 1 M HCl per 1 mL of sample. The reaction mixture after filtration was extracted three times with chloroform. Using authentic standards the products of biotransformations were identified by GC using a Thermo-Quest-Finnigan (Plymouth, Minnesota, USA) instrument equipped with an RTX 1 column with a

flame ionization detector (FID). Nitrogen was used as the carrier gas at a constant flow of 1.5 mL min^{-1}, and then held for 5 min. The injector and detector (FID) temperature were kept at 150° and 270°C, respectively. One unit of enzyme activity was defined as the amount of enzyme which converted 1 μmol of substrate per minute under the assay conditions.

Results and discussion

The reaction was carried out at different pH values and two substrate concentrations (10 and 50 mM). At 50 mM, this organism retained its bioconversion ability but with reduced yields and prolonged reaction times (Table I) probably due to substrate or product inhibition of enzyme activity. As a result further studies on the biocatalytic performance of *Aspergillus* sp. PTCC 5266 were performed using nitrile concentration of 10 mM. The enzyme activity was tested, under identical conditions, after 2 to 7 days of incubation (Table II). Although the enzyme retained its activity almost completely for 4 days, the activity subsequently reduced, resulting in low yields (especially for aliphatic nitriles, entries 27, 31 and 35).

Table I. Biotransformation of nitriles by whole cells of *Aspergillus* sp. PTCC 5266[a].

Entry	Substrate (initial concentration, mM)	Reaction time (h)	Product	Yield (%)
1	**1a** (10)	4	**1b**	80
2			**1c**	17
3	**1a** (50)	8	**1b**	79
4			**1c**	12
5	**2a** (10)	5	**2b**	71
6			**2c**	21
7	**2a** (50)	8	**2b**	79
8			**2c**	11
9	**3a** (10)	0.5	**3b**	72
10			**3c**	24
11	**3a** (50)	5	**3b**	81
12			**3c**	13
13	**4a** (10)	30	**4b**	83
15	**4a** (50)	48	**4b**	72
17	**5a** (10)	30	**5b**	94
19	**5a** (50)	72	**5b**	86
21	**6a** (10)	24	**6b**	64
23	**6a** (50)	72	**6b**	53
25	**7a** (10)	24	**7b**	67
27	**7a** (50)	144	**7b**	59
29	**8a** (10)	72	**8b**	25
31	**8a** (50)	120	**8b**	9
33	**9a** (10)	72	**9b**	32
35	**9a** (50)	168	**9b**	8

[a]Biotransformations of nitriles (Scheme 1) in two concentrations were carried out in a reaction mixture (total volume 100 mL) comprising sodium phosphate buffer (50 mM, pH 7.0), whole cells of *Aspergillus* sp. PTCC 5266 corresponding to 3 mg dry cell weight/mL and dimethylsulfoxide (2%, v/v) as co-solvent. Substrates and products were determined by GC and comparison with authentic standards.

Table II. Residual enzyme activity after 7 days of incubation (in sodium phosphate buffer (50 mM, pH 7.0).

Incubation time (days)	Enzyme activity (%)
1	100
2	99
3	99
4	98
5	94
6	91
7	87

The influence of pH over the range pH 5–10 on the conversion of all substrates at 10 mM concentration and 26°C was investigated (Figure 1). Nitrile-hydrolyzing activity was not evident at pH ≤ 5 and the optimum pH for all reactions turned out to be 7.0. It is noteworthy that the experiments showed the pH for maximal activity also coincided with that for maximal biocatalyst stability. This is typical for most conversions of nitriles to corresponding amides and carboxylic acids (Graham et al. 2000; Šnajdrová et al. 2004; Kaplan et al. 2006a; Raj et al. 2007). A few biocatalysts have been reported to be active over a broad pH range, such as *Rhodococcus* sp. (between pH 3 and 10) (Kato et al. 1999) and polyvinyl alcohol-entrapped *Rhodococcus* sp. whole cells (between pH 2 and 13) (Bauer et al. 1996). Rustler and co-workers investigated the acid tolerance of whole cells of *Exophiala oligosperma* R1 (Rustler & Stolz 2007). The cells could convert phenylacetonitrile at pH 4, which was lower than the value determined for *Escherichia coli* cells. Resting cells of *E. oligosperma* were incubated at pH 1.5–9 with phenylacetonitrile. These experiments showed that the cells converted phenylacetonitrile over the whole pH range and no spontaneous hydrolysis of phenylacetonitrile was observed under acidic reaction conditions in control experiments without cells (Rustler et al. 2007).

Most of the nitriles examined in the present study were metabolized by nitrilase to the corresponding carboxylic acids, while three nitriles (4-nitrophenylacetonitrile **1a**, 2-chlorobenzonitrile **2a** and 3-chlorobenzonitrile **3a**) were transformed to the corresponding carboxylic acids and amides (**1b** + **1c**, **2b** + **2c** and **3b** + **3c**, with relatively high acid to amide ratio). 4-Nitrophenylacetonitrile **1a** was the best substrate of those tested and the total conversions (carboxylic acid + amide) were 97% and 91% in 10 mM and 50 mM concentrations, within 4 and 8 h, respectively. A comparison between 2-chlorobenzonitrile **2a** and 3-chlorobenzonitrile **3a** showed that the latter had a higher conversion (96%, 94% versus 92%, 90%) in a shorter reaction time (0.5 h, 5 h versus 5 h, 8 h) at 10 mM and 50 mM,

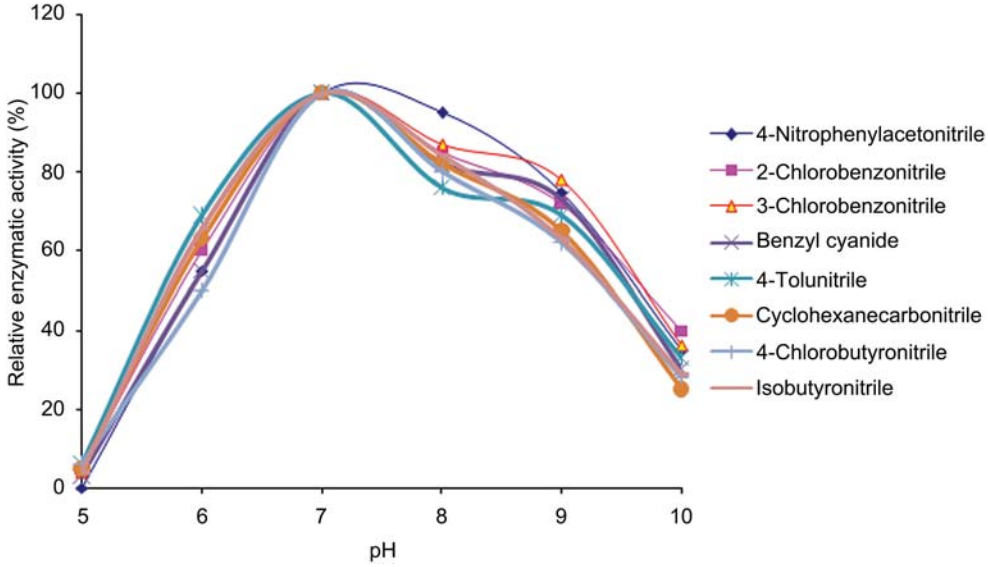

Figure 1. Effect of pH on the nitrile-hydrolyzing activity of whole cells of *Aspergillus* sp. PTCC 5266 (10 mM). Activity was assayed at 26°C and average dry cell weight of 300 mg corresponding to 10 g wet cell weight; activity of whole cells at pH 7 was taken as 100%.

respectively. The slow reaction of the *ortho*-substituted benzonitrile **2a** (8 h) compared with *meta*-chlorobenzonitrile **3a** (0.5 h) is probably due to the steric hindrance in the enzyme active site for the former. Table 1 illustrates that the production of amides in addition to carboxylic acids was especially pronounced for substrates with electron-withdrawing groups. The other organonitriles – three aromatic nitriles, i.e. benzyl cyanide **4a**, benzonitrile **5a** and 4-tolunitrile **6a**; and three aliphatic nitriles, i.e. cyclohexanecarbonitrile **7a**, 4-chlorobutyronitrile **8a** and isobutyronitrile **9a** – were transformed only to their corresponding carboxylic acids (**4b**–**9b**) by nitrilase. No other products were detected. As illustrated in Table 1, benzonitrile **5a** was the best aromatic nitrile for this organism with 94 % and 86% conversion at the two different concentrations. Among the aliphatic nitriles, the cyclic nitrile cyclohexanecarbonitrile **7a** gave higher yields compared with the acyclic nitriles **8a** and **9a**, which had the lowest yields among all the nitriles.

Within the past few years, various aspects of nitrilases have been reviewed (Martínková et al. 2008, 2009b; Malandra 2009; Winkler et al. 2009; Vejvoda et al. 2010). Genes encoding putative nitrilases have been found mostly in *Aspergillus*, *Gibberella* and *Penicillium*. Fungal nitrilases have high specific activity towards substrates such as benzonitrile and analogues, 3- and 4-cyanopyridine and also some aliphatic nitriles of medium chain length, leading to classification as aromatic nitrilases (Martínková & Křen 2010). Minor activities were found for substituted and branched substrates like lactonitrile, isobutyronitrile, 2-chloropropionitrile and 2-phenylpropionitrile (Martínkova et al. 2009b).

Aspergillus sp. PTCC 5266 accepted both aliphatic and aromatic nitriles and could be used for the production of carboxylic acids with relatively high yields. Some modification in the reaction conditions may improve the yields and acid/amide ratio.

Amide synthesis

The hydration of nitriles to amides has been often observed as a side reaction catalyzed by nitrilase, but it has not been studied in detail until recently (Fernandes et al. 2006). It occurs mainly with substrates bearing electron-withdrawing and bulky substituents (Martínková & Křen, 2010).

Detection of amide is not good evidence for the presence of a nitrile hydratase/amidase pathway as amide production catalyzed by nitrilases has previously been observed (Šnajdrová et al. 2004). The nitrilase from *Aspergillus niger* (Kaplan et al. 2006a) afforded high amounts of amides especially from substrates with electron-withdrawing groups. Amides made up 4–6% of the total products in hydrolysis of nitriles by the nitrilase from *Fusarium oxysporum* f. sp. melonis (Goldlust & Bohak 1989), while the nitrilase from *Rhodococcus* ATCC 39484 also formed phenylacetamide from phenylacetonitrile. Stevenson et al. (1992) proposed that a tetrahedral intermediate is formed which, for some substrates, can break down anomalously to produce amide in place of the normal acid product.

For further investigation on nitrilase activity and to differentiate between nitrilase and nitrile hydratase/amidase activity, the biocatalyst was incubated with benzamide. However, it was found that *Aspergillus* sp. PTCC 5266 was unable to convert benzamide to

benzoic acid under the reaction conditions adopted. Although it is known that the activity of nitrile hydratase/amidase systems can be selectively controlled through the appropriate choice of operational conditions, such as in *Microbacterium imperiale* (Cantarella et al. 2006), current evidence suggests that *Aspergillus* sp. PTCC 5266 contains a nitrilase only. Amide production using nitrilase on substrates with electron-withdrawing groups is a common characteristic and the pH profiles for all of the substrates were similar. Furthermore, nitrile hydratases have hitherto not been detected in fungi.

Our biocatalyst produced a high acid/amide ratio for 4-nitrophenylacetonitrile, 2-chlorobenzonitrile and 3-chlorobenzonitrile, and only carboxylic acid for the other substrates, so it may be useful for synthesis of carboxylic acids. The formation of amides may complicate the use of nitrilases for carboxylic acid production. However, the benefit of nitrilases in comparison with nitrile hydratases is their higher stability and enantioselectivity (Martínková & Křen, 2010).

Conclusions

Aspergillus sp. PTCC 5266 transforms nitriles, especially aromatic nitriles, probably via a nitrilase pathway. The product of the biotransformation of most substrates was the pure carboxylic acid, but some nitriles gave a low ratio of the corresponding amide. The *Aspergillus* sp. PTCC 5266 nitrile-hydrolyzing activity exhibited significant stability over prolonged reaction times compared with previously described fungal nitrile-hydrolyzing microorganisms. Also it accepts both aliphatic and aromatic nitriles and could be used for producing carboxylic acids with relatively high yields.

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