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Article

Nitrile Hydratase Activity of *Nocardia corallina* B-276

Herminia I. Pérez*, Norberto Manjarrez, Héctor Luna, Aída Solís and Concepción Ramírez

Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana Unidad Xochimilco, Calz. del Hueso No. 1100, Col. Villa Quietud, Deleg. Coyoacan, 04960 México D. F., México

A atividade da nitrila hidratase de *Nocardia corallina* B-276 é descrita. O único produto detectado, na hidrólise biocatalisada das nitrilas selecionadas, foi a amida correspondente. O melhor resultado foi obtido usando-se suspensão de células em tampão de fosfatos (pH = 7,0), com porcentagens de conversão na faixa de 31% a > 99%.

The nitrile hydratase activity of *Nocardia corallina* B-276 is described, and the only detected product during the biocatalyzed hydrolysis of selected nitriles was the corresponding amide. The best results were obtained using a suspension of cells in a phosphate buffer (pH= 7.0), with conversion percentages ranging from 31% to > 99%.

Keywords: nitrile hydratase, biotransformation, nitriles, amides, *Nocardia corallina*

Introduction

Nitrile is a functional group that usually is transformed to amides or carboxylic acids under strong reaction conditions in acidic or basic media and high temperatures. Amides have also been prepared from nitriles at room temperature using strong oxidizing agents such as hydrogen peroxide[1] or sodium superoxide in DMSO.[2] On the other hand biocatalytic hydrolysis of nitriles mediated by nitrilase, nitrile hydratase (NHase) or amidase, is an important option for these processes.[3] In general, these bioconversions, are fast under mild reaction conditions, there are no formation of secondary products, contamination due to chemical side reactions can be avoided or diminished, and the reaction can be regio- and stereoselective, and thus it is an important alternative for the preparation of chiral compounds.

These biocatalysts have important applications in industry for the enantioselective preparation of amides, for the transformation of waste nitrile to the less toxic amides or carboxylic acids. In Japan acrylamide is produced by the hydrolysis of acrylonitrile using resting cells of *Rhodococcus rhodochrous* J1;[4, 5] and 2-arylpropionitriles have been enantioselectively hydrolyzed to the corresponding amides or carboxylic acids using the bacteria of the genera *Rhodococcus*,[6-9] *Agrobacterium*,[9,10] and *Pseudomonas.*[11]

* e-mail: hperez@correo.xoc.uam.mx

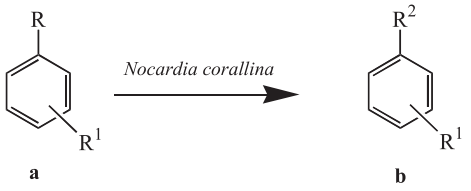
Microorganisms of the genera *Rhodococcus* are some of the most common sources of NHases and amidases[12] and, due to the fact that some authors have taxonomically classified *Nocardia corallina* B-276 (bacteria which we have used previously in reactions of oxidation[13-16]) as *Rhodococcus corallinus*,[17] we decided to study the possibility that *Nocardia corallina* B-276 could be used as a biocatalyst to hydrolyze nitriles.

Results and Discussion

The nitriles used to explore their possible hydrolysis using *Nocardia corallina* as biocatalyst were benzonitrile (**1a**), 4-chlorophenylacetonitrile (**2a**), phenylacetonitrile (**3a**), 4-methoxybenzonitrile (**4a**) and 3,4-dimethoxyphenylacetonitrile (**5a**), (Scheme 1). Nitriles are known as potential raw materials for fine chemistry.[3]

Also, the selection of the aromatic compounds that have substituents in the *meta* and *para* position of the ring is important, because NHases are metalloenzymes that can coordinate better with these compounds.[18] Another important structural fact is that during the oxidations using *N. corallina*, we have observed an important steric effect in the case of aromatic rings with *ortho* substituents.[13-16]

The biotransformation of nitriles by *N. corallina* was carried out using two methods. In method A we use the cells in the culture media and in method B we use the resting cells suspended in a phosphate buffer (pH= 7.0).



a b

	R	R^1	R^2
1	CN	H	$CONH_2$
2	CH_2CN	4-Cl	CH_2CONH_2
3	CH_2CN	H	CH_2CONH_2
4	CN	4-OCH_3	$CONH_2$
5	CH_2CN	3,4-diOCH_3	CH_2CONH_2

Scheme 1.

The hydrolysis of benzonitrile (**1a**, entry 1, Table 1) using method A gave the product with an isolated yield of 26%, after 30 h of reaction, and this method also has the disadvantage that the isolation of the corresponding benzamide was difficult due to its high solubility in water. To achieve better results, we decided to use resting cells of *N. corallina* and, as can be observed in Table 1 (entries 2 – 5), an increase in the cell ratio improved substantially the conversion degree. For example, when the ratio changed from 1:40 to 1:100 the conversion was greatly improved, from 44 to 89%, meaning that the conversion degree was doubled. The corresponding benzoic acid was not detected during the biotransformation using any of the two methods.

To determine the possible hydrolysis of benzamide (**1b**) to benzoic acid under the conditions of method B, the reaction was performed with a ratio substrate:cells of 1:40, and after 48 h the corresponding carboxylic acid was not detected by HPLC, and the amide **1b** remained unchanged.

Table 1. Biotransformation of benzonitrile **1a**

Entry	Method	m substrate: m wet cells	Reaction time (h)	Conversion%
1	A	Culture of cells[a]	30	26[b]
2	B	1:40	48	44[c]
3	B	1:60	48	58[c]
4	B	1:80	48	67[c]
5	B	1:100	48	89[c]

[a] 0.85-1.45 mmol of substrate were used and the quantity of cells in the culture medium were not determined; [b] Isolated yield; [c] Determined by HPLC. Reaction conditions were not optimized.

In the case of 4-chlorophenylacetonitrile (**2a**) hydrolysis using method A, and after 118 h, the isolated yield of amide was only 11% (entry 1, Table 2). Also, in this case the results of the biocatalyzed hydrolysis under the reaction conditions of method B were much better. The biotransformation of **2a** to the corresponding amide **2b**, with a ratio of 1:400 gave a 90% conversion in 24 h, with an increase of only 6% after 48 h (entries 2 and 3, Table 2). Similar results were obtained using a ratio of 1:200 (entries 4 and 5, Table 2). In contrast with the hydrolysis of benzonitrile (**1a**) to benzamide (**1b**) that needed a substrate:cells ratio of 1:100 to get a conversion of 89% (entry 5**,** Table 1), for 4-chlorophenylacetamide (**2a**) using a lower ratio 1:15, the conversion was quantitative after 89 h (entry 6, Table 2). Once again the corresponding carboxylic acid was not detected by IR or TLC, under any of the reaction conditions using the two methods.

Table 2. Biotransformation of 4-chlorophenylacetonitrile **2a**

Entry	Method	m substrate: m wet cells	Reaction time (h)	Conversion%
1	A	Culture of cells[a]	118	11[b]
2	B	1:400	24	90[c]
3	B	1:400	48	96[c]
4	B	1:200	24	90[c]
5	B	1:200	48	93[c]
6	B	1:15	89	>99[c]

[a] 0.85-1.45 mmol of substrate were used and the quantity of cells in the culture medium were not determined; [b] Isolated yield; [c]Determined by HPLC; Reaction conditions were not optimized.

The hydrolysis of nitriles **3a**, **4a** and **5a** to the corresponding amides resulted in very low conversions under method A, even after 192 h. Most of the starting material was recovered unchanged, and similar results were obtained for nitriles **1a** and **2a**.

Contrary to what was expected, and in spite of a high substrate: cells ratio 1:200, after 48 h the hydrolysis of phenylacetonitrile (**3a)** to the amide **3b** was 31% (entry 2, Table 3).

Better results were achieved in the hydrolysis of 4-methoxybenzonitrile (**4a**) and 3,4-dimethoxyphenylacetonitrile (**5a**). With a substrate:cells ratio 1:200 after 48 h, 53% conversion was obtained for 4-methoxybenzamide (**4b**) and 52% for 3,4-dimethoxyphenylacetamide (**5b**) (Table 3). The corresponding carboxylic acids were not detected in any case.

Table 3. Biotransformation of nitriles **3a**, **4a** and **5a** with resting cells of *Nocardia corallina*

Entry	Compound	m substrate: m wet cells	Reaction time (h)	Conversion% [a]
1	**3**	1:200	24	27
2	**3**	1:200	48	31
3	**4**	1:200	48	53
4	**5**	1:200	48	52

[a]Determined by HPLC. Reaction conditions were not optimized.

To the best of our knowledge, this is the first report about the use of *N. corallina* B-276 for the hydrolysis of nitriles to amides, and it is worth mentioning that the

hydrolysis continues until the amide, and is not further hydrolyzed to the carboxylic acid under the reaction conditions of methods A and B. This is important because amides are very useful intermediates and, in many cases are difficult to prepare,[19] since they are more easily hydrolyzed to the carboxylic acid than the nitrile itself. It has been reported that microorganisms of the genera *Nocardia* and *Rhodococcus* are able to hydrolyze nitriles to amides but frequently the reaction cannot be stopped in the amide stage and a mixture of amide and acid or only the acid is obtained.[20] This is not the case of *N. corallina* B-276 which can produce only amides selectively, in high yields depending on the experimental conditions and substrate structure. Then the use of an amidase inhibitor is not necessary.[21]

Experimental

Nitriles were prepared by conventional methods (**2a**)[22] or purchased from Aldrich (**1a, 3a, 4a, 5a**). Nitriles and amides were identified by their infrared spectra (Perkin-Elmer Paragon 1600 FT), as liquid films or KBr discs; Proton Nuclear Magnetic Resonance (Varian 400 MHz); and by TLC on silica gel 60 GF_{254} Merck, by comparative analysis with authentic samples, column chromatography using flash chromatography silica gel (230-240 mesh Merck). HPLC analysis was performed on an Agilent 1100 series liquid chromatograph, equipped with a diode array detector, using Hypersil BDS-C18 (5μm, 250 X 4.6 mm) column. The mobile phase was methanol-water (80:20), 0.8 mL min^{-1} and 24°C.

Organism and growth[23]

Nocardia corallina B-276 (ATCC 31338) was grown at 28-30°C on agar plates (15 g L^{-1} agar; 3 g L^{-1} beef extract; 5 g L^{-1} peptone;).

Liquid cultures were incubated in an orbital shaker, the broth composition was: *Solution A*. 0.05 g L^{-1} $FeSO_4{\cdot}7H_2O$; 1.74 g L^{-1} K_2HPO_4; 2 g L^{-1} $(NH_4)_2SO_4$; 1 g L^{-1} yeast extract; *Solution B*. 1.5 g L^{-1} $MgSO_4$; *Solution C*. 2 g L^{-1} glucose; each solution was sterilized separately, later combined and the pH adjusted to 8.0 (± 0.5). The biocatalyzed hydrolysis of nitriles was carried out following two methods: method A and method B.

Method A: biotransformation using cells in culture medium[13]

Pre-culture I. A 125 mL Erlenmeyer flask containing 50 mL of sterile culture medium was inoculated from an agar plate (three days old) and incubated at 28-30 °C on an orbital shaker (170 rpm) for 20-24 h.

Pre-culture II. The content of the pre-culture I flask was aseptically poured into a 250 mL Erlenmeyer flask containing 100 mL of fresh sterile culture medium. The flask was incubated at 28-30 °C on an orbital shaker (170 rpm) for 24 h.

Biotransformation. Under aseptic conditions the substrate (0.85-1.45 mmol), in 1 mL of *N,N*-dimethyl-formamide, was added to the flask containing pre-culture II, followed by the addition of *n*-octane (10 mL). The reaction mixture (161 mL final volume) was incubated at 28-30 °C on an orbital shaker (170 rpm). The progress of the biotransformation was then monitored by TLC, and stopped at the time indicated for each substrate, the reaction mixture was saturated with NaCl and filtered through Celite, the pH was adjusted to 10-10.5 with aqueous NaOH (2 mol L^{-1}). Amides and unreacted nitrile were extracted with ethyl acetate (4 x 25 mL), and after drying over anhydrous sodium sulfate the solvent was evaporated under vacuum. The aqueous phase was then acidified to pH 2 with aqueous HCl (0.5 mol L^{-1}), and the acids were extracted with CH_2Cl_2 (4 x 25 mL) after drying over anhydrous sodium sulfate, and the solvent was then evaporated under vacuum. The products were purified by flash chromatography.

Method B: biotransformation using resting cells[24]

Pre-culture. A 500 mL Erlenmeyer flask containing 200 mL of sterile culture medium was inoculated from an agar plate (three days old) and incubated at 28-30 °C on an orbital shaker (170 rpm) for 48 h. The cells were collected by centrifugation at 4500 rpm for15 min. Cells were washed twice with potassium phosphate buffer (0.1 mol L^{-1}, pH 7.0).

Biotransformation. The wet cells were incubated in 50 mL phosphate buffer, pH 7.0, for 30 min at 28-30 °C on an orbital shaker (170 rpm), the substrates were added in the substrate:cells ratio (m/m) as indicated in each case and were shaken under the same conditions for 20-48 h, depending on the substrate. The reaction mixture was then centrifuged at 4500 rpm for 15 min. Samples were analyzed by HPLC, using a C-18 column, and MeOH-H_2O (80:20) as the eluent, to determine the conversion degree.

Conclusions

These results demonstrate that *N. corallina* B-276 has the ability to hydrolyze nitriles to amides, and because the corresponding carboxylic acid was not detected in any case,

we assume that the enzymes responsible of this activity are the nitrile hydratases. The NHase activity of *N. corallina* B-276 was higher using isolated cells suspended in phosphate buffer in comparison to the culture medium.

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