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# Biotransformation of nitriles to amides using soluble and immobilized nitrile hydratase from *Rhodococcus erythropolis* A4

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## Abstract

A semi-purified nitrile hydratase from *Rhodococcus erythropolis* A4 was applied to biotransformations of 3-oxonitriles **1a–4a**, 3-hydroxy-2-methylenitriles **5a–7a**, 4-hydroxy-2-methylenitriles **8a–9a**, 3-hydroxynitriles **10a–12a** and 3-acyloxynitrile **13a** into amides **1b–13b**. Cross-linked enzyme aggregates (CLEAs) with nitrile hydratase and amidase activities (88% and 77% of the initial activities, respectively) were prepared from cell-free extract of this microorganism and used for nitrile hydration in presence of ammonium sulfate, which selectively inhibited amidase activity. The genes *nha1* and *nha2* coding for  $\alpha$  and  $\beta$  subunits of nitrile hydratase were cloned and sequenced.

**Keywords:** *Rhodococcus erythropolis*; Nitrile hydratase; Amidase; Branched and substituted nitriles; Cross-linked enzyme aggregates

## 1. Introduction

Bacterial cells with nitrile hydratase activity have been widely used to produce amides from nitriles [1]. However, whole cells usually transform the amides further into carboxylic acids and ammonia by amidases, which are expressed along with nitrile hydratases from the same operon. In this way, the yield and purity of the amide are deteriorated. Therefore, a search for selective amidase inhibitors was performed and dimethylsulfoxide and molecular iodine were shown to assist the preferential action of the nitrile hydratase [2]. Diethylphosphoramidate was also proposed as a selective amidase inhibitor [3] and used for the synthesis of 3-oxoamides from the corresponding nitriles by *Rhodococcus rhodochrous* whole cells [4]. Alternatively, it is possible to control the enzyme activities by reaction conditions, as demonstrated for nitrile hydratase/amidase enzyme system in *Microbacterium imperiale* [5].

The use of purified or partially purified nitrile hydratases appears to be another solution to this problem. However, this approach was hampered by instability of the enzymes and, until recently, their inavailability on the market. In the present work we demonstrated utility of a semi-purified nitrile hydratase from *Rhodococcus erythropolis* A4 (formerly *Rhodococcus equi* A4) [6] for amide production from branched and substituted nitriles. As a cheaper and less laborious alternative, a cell-free extract was used, in which the amide hydrolysis was slowed down by ammonium sulfate, which was previously reported as an efficient inhibitor of the amidase in strain A4 [7]. In order to simplify handling of the nitrile hydratase and to improve its stability, we searched for a suitable immobilization method. Recently, an innovative immobilization technique, which resided in precipitation of the enzyme by different additives like salts or organic solvents and cross-linking of the resulting physical aggregates with glutaraldehyde (CLEAs) [8], was successfully applied to a number of different enzymes [9]. A modification of this method using a milder cross-linking agent, dextran polyaldehyde, was also suitable for the immobilization of relatively labile enzymes like nitrilases [10]. Therefore, we examined the suitability of

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this method for immobilization of the nitrile hydratase from *R. erythropolis* A4.

## 2. Experimental

### 2.1. Chemicals

3-Oxo-3-phenylpropanenitrile (**1a**), 3-(3-chlorophenyl)-3-oxopropanenitrile (**2a**) and 3-(4-chlorophenyl)-3-oxopropanenitrile (**3a**) were purchased from Avocado and 4,4-dimethyl-3-oxopentanenitrile (**4a**) from Aldrich. (*R,S*)-2-(1-Hydroxyethyl)propenenitrile (**5a**), (*R,S*)-2-(1-hydroxybutyl)propenenitrile (**6a**), (*R,S*)-2-( $\alpha$ -hydroxybenzyl)propenenitrile (**7a**), (*R,S*)-4-hydroxy-2-methylenehex-5-enenitrile (**8a**) and (*R,S*)-2-(2-hydroxy-3,3-dimethoxypropyl)propenenitrile (**9a**) were prepared according to literature procedures [11–13]. (*R,S*)-3-Hydroxy-3-phenylpropanenitrile (**10a**) and (*R,S*)-3-hydroxy-4,4-dimethylpentanenitrile (**11a**) were obtained by conventional  $\text{NaBH}_4$ -reduction of **1a** and **4a**, respectively. (*R,S*)-3-(2-Furyl)-3-hydroxypropanenitrile (**12a**) was obtained by  $\text{NaBH}_4$ -reduction of 3-(2-furyl)-3-oxopropanenitrile, which, in turn, was prepared by an adaptation of a published method [14]. Briefly, acetonitrile was reacted with sodium amide in diethyl ether at  $-30^\circ\text{C}$  and then with ethyl 2-furoate (Aldrich) under the same conditions until the ester disappeared. (*R,S*)-3-(Benzoyloxy)hexanenitrile **13a** was prepared via an intermediate (3-hydroxyhexanenitrile), which was synthesized in an analogous way as **12a** (from sodium amide and ethyl butyrate), followed by overnight reaction with 3 equiv. of benzoyl chloride in the presence of 1.3 equiv. of pyridine and catalytic amounts of 4-dimethylaminopyridine in dichloromethane at  $0^\circ\text{C}$  to room temperature. Dextran polyaldehyde was prepared by oxidation of dextran as described previously [10].

### 2.2. Microorganism and cultivation

*R. erythropolis* A4 (formerly denoted as *R. equi* according to biochemical tests, re-classified according to 16S rDNA sequencing [15]; deposited in the Culture Collection of Microorganisms, Masaryk University, Brno, Czech Republic) was grown in 500-mL flasks with 100 mL of mineral medium [16] supplemented with yeast extract (3 g/L) and acetonitrile (15 mM). The cultivation proceeded at shaking (200 rpm) and  $28^\circ\text{C}$  for 2 days.

### 2.3. Preparation of cell-free extract

Whole cells of *R. erythropolis* A4 were disintegrated by milling with glass beads as described previously [7].

### 2.4. Enzyme purification

Semi-purified nitrile hydratase from *R. erythropolis* A4 was prepared as described previously [6] and stored at  $-80^\circ\text{C}$  in Tris/HCl buffer (50 mM, pH 7.5) containing approximately 0.5 M ammonium sulfate.

### 2.5. Immobilization in cross-linked enzyme aggregates

Precipitant screening was performed as described previously [9]. Briefly, 0.010 mL of enzyme solution (approximately 0.1 U) was added to 0.090 mL precipitant – 70% saturated solution of ammonium sulfate, *tert*-butyl alcohol, acetone, acetonitrile, ethyl lactate or polyethylene glycol (relative molecular weight 1305–1595) – and after 15 min at room temperature the precipitate was redissolved in 0.9 mL of Tris/HCl buffer (50 mM, pH 8). Nitrile hydratase activities were assayed as described below. For the production of CLEAs, 20 mL of cell-free extract (approximately 45 mg of protein) was added to 47 mL of saturated ammonium sulfate (with 1 mM EDTA, pH 8.0). Dextran polyaldehyde (3% solution, 4 mL) was added and the mixture was stirred for 45 min at  $4^\circ\text{C}$ . After centrifugation, CLEAs were treated with 0.2% sodium borohydride dissolved in saturated sodium bicarbonate, centrifuged again, washed three times with Tris/HCl buffer (50 mM, pH 8) and stored at  $4^\circ\text{C}$ .

### 2.6. Activity assay

The nitrile hydratase was assayed in a reaction mixture (0.5 mL) containing 25 mM benzonitrile, Tris/HCl buffer (50 mM, pH 8) and an appropriate amount of the soluble enzyme or CLEAs (approximately 0.25 U). The reaction proceeded for 10 min at  $30^\circ\text{C}$  with shaking in Thermomixer Comfort Eppendorf (850 rpm) and was stopped with 0.1 mL of 1 M HCl. After centrifugation of the precipitated protein, benzonitrile, benzamide and benzoic acid were determined by HPLC (see below).

### 2.7. General procedure for biotransformations

The reactions proceeded at  $28^\circ\text{C}$  with shaking in Na/K phosphate buffer (50 mM, pH 8) containing 2.5–25 mM substrate, methanol (2–5%, v/v) as cosolvent and an appropriate amount of the semi-purified enzyme (see Tables 1–3). Alternatively, the reactions were catalyzed by CLEAs (see Table 4) in absence or presence of ammonium sulfate (360–800 mM) and 2 mM EDTA. The reactions were monitored by analytical HPLC (see below).

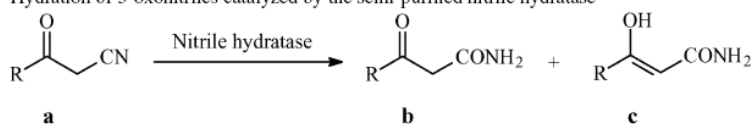
### 2.8. Determination of nitrile hydratase stability

Cell-free extract or CLEAs were incubated in 50 mM Tris/HCl buffer, pH 8, at  $28^\circ\text{C}$  with shaking in Thermomixer Comfort Eppendorf (850 rpm). At intervals, samples were withdrawn and assayed for nitrile hydratase activity as described above.

### 2.9. HPLC analysis

Nitriles and biotransformation products were determined by reversed-phase HPLC with a Nova-Pak C<sub>18</sub> column (5  $\mu\text{m}$ , 3.9 mm  $\times$  150 mm) and mobile phase consisting of 10% acetonitrile (compounds **5a–6a**, **8a–12a** and their products), 25% acetonitrile (compounds **1a–4a**, **7a** and their products), 30% acetonitrile (benzonitrile and its products) or 40% acetonitrile (compounds **13a**, **13b**) and 0.1% phos-

Table 1  
Hydration of 3-oxonitriles catalyzed by the semi-purified nitrile hydratase

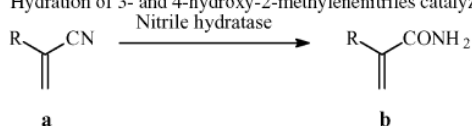


Entry	Substrate (mM)	R	Reaction time (min)	Enzyme/substrate ratio (U <sup>a</sup> /mmol substrate)	Substrate conversion (%)	Product	Isolated yield (%) <sup>b</sup>
1	<b>1a</b> (10)	Phenyl	60	85	>99	<b>1b + 1c</b>	62
2	<b>2a</b> (10)	3-Chlorophenyl	60	85	>99	<b>2b + 2c</b>	95
3	<b>3a</b> (10)	4-Chlorophenyl	60	85	>99	<b>3b + 3c</b>	86
4	<b>4a</b> (10)	<i>tert</i> -Butyl	60	85	>99	<b>4b + 4c</b>	91

<sup>a</sup> Activity determined with benzonitrile (25 mM) as substrate.

<sup>b</sup> On 20–30 mg scale.

Table 2  
Hydration of 3- and 4-hydroxy-2-methylenitriles catalyzed by the semi-purified nitrile hydratase



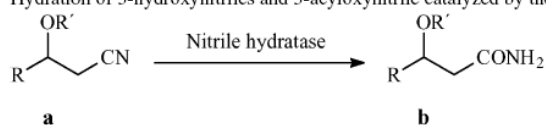
Entry	Substrate (mM)	R	Reaction time (min)	Enzyme/substrate ratio (U <sup>a</sup> /mmol substrate)	Substrate conversion (%)	Product	Isolated yield (%) <sup>b</sup>
5	<b>5a</b> (25)		30	6	>99	<b>5b</b>	82
6	<b>6a</b> (25)		120	10	>99	<b>6b</b>	79
7	<b>7a</b> (25)		180	10	>99	<b>7b</b>	69
8	<b>8a</b> (25)		60	6	>99	<b>8b</b>	67
9	<b>9a</b> (2.5)		1200	20	74	<b>9b</b>	27 <sup>c</sup>

<sup>a</sup> Activity determined with benzonitrile (25 mM) as substrate.

<sup>b</sup> On scale of 25 mg (**10a**) or approximately 70 mg (other compounds).

<sup>c</sup> Purified by preparative HPLC.

Table 3  
Hydration of 3-hydroxynitriles and 3-acyloxynitrile catalyzed by the semi-purified nitrile hydratase



Entry	Substrate	R	R'	Reaction time (min)	Enzyme/substrate ratio (U <sup>a</sup> /mmol substrate)	Substrate conversion (%)	Product	Isolated yield <sup>b</sup> (%)
10	<b>10a</b> (15)	Phenyl	H	60	10	>99	<b>10b</b>	82
11	<b>11a</b> (15)	<i>tert</i> -Butyl	H	60	25	>99	<b>11b</b>	91
12	<b>12a</b> (10)	2-Furyl	H	60	20	>99	<b>11b</b>	58
13	<b>13a</b> (2.5)	Propyl	Bz	180	130	>99	<b>13b</b>	79

<sup>a</sup> Activity determined with benzonitrile (25 mM) as substrate.

<sup>b</sup> On 15–25 mg scale.

Table 4

Conversion of 3-oxo-3-phenylpropanenitrile (**1a**) and (*R,S*)-2-(1-hydroxyethyl)propanenitrile (**5a**) by cross-linked nitrile hydratase/amidase aggregates (0.4 U mL<sup>-1</sup> reaction mixture)

Entry	Substrate (mM)	Ammonium sulfate (mM)	Reaction time (min)	Nitrile conversion (%) <sup>a</sup>	Amide yield (%) <sup>a</sup>	Ratio of amide in total product (%) <sup>a</sup>
14	<b>1a</b> (10)	0	180	>99	3.2	3.2
15		360	180	>99	75.3	75.3
16		800	180	71.3	65	86.9
17	<b>5a</b> (10)	0	180	>99	42.9	42.9
18		360	180	>99	66.9	66.9
19		800	180	92.3	81.8	88.6

Note: The remainder of total product consisted of 3-oxo-3-phenylpropanoic acid (**1d**) and acetophenone (entries 14–16) or (*R,S*)-2-(1-hydroxyethyl)propanoic acid (**5c**, entries 17–19).

<sup>a</sup> Determined by HPLC.

phoric acid in water at a flow rate of 0.9 mL min<sup>-1</sup>. Enantiomers of **6b**, **7a**, **7b** and **9a** were separated using Chiralcel OD-H column (Daicel) with a mobile phase consisting of hexane: 2-propanol, 9:1 (v/v) at a flow rate of 0.8 mL min<sup>-1</sup>. Separation of enantiomers of **10a** was performed using Chiral-AGP column (5 μm particles, 100 mm × 4.0 mm, ChromTech, Hägersten, Sweden) with a mobile phase consisting of 10 mM Na-phosphate buffer, pH 7.0, and 1 mM *N,N*-dimethyloctylamine at a flow rate of 0.9 mL min<sup>-1</sup>.

### 2.10. Product isolation and identification

Amides were extracted from the reaction mixtures with ethyl acetate at pH 8.5–9 (1 M KOH) or from the lyophilisates of the reaction mixtures with dry methanol (compounds **5b–6b**). Compound **9b** was purified by preparative HPLC using a Nucleosil 120-5, C<sub>18</sub> column (5 μm, 8 mm × 250 mm; Macherey-Nagel) and a mobile phase consisting of 10% acetonitrile and 0.1% acetic acid in water at a flow rate of 1 mL min<sup>-1</sup>. The products were characterized by their <sup>1</sup>H and <sup>13</sup>C NMR spectra (recorded on a 400 MHz spectrometer Varian UNITY Inova-400).

### 2.11. Cloning and sequencing

The DNA manipulation techniques and transformation of *R. erythropolis* were carried out as described previously [17]. To amplify the DNA fragment covering parts of the nitrile hydratase genes (coding for its α- and β-subunits) by PCR, the degenerated primers NHAF1 (GTGTGCT/ACG/TCTGTGTTC) and NHBR2 (TCGTGT/GAC/TTCCATCCAT) designed according to the conserved regions of the sequences of genes coding for Fe-type nitrile hydratases from several *Rhodococcus* strains were used: *R. erythropolis* AJ270 (GenBank accession no. AJ716152), *R. erythropolis* DSM43006 (AY223835), *R. erythropolis* ARG-AN024 (AY223830), *R. erythropolis* ANT-AN007 (AY223827), *R. globerulus* A-4 (AB105912), *R. rhodochrous* L (E03848), *R. rhodochrous* H (E03847) and *Rhodococcus* sp. N-771 (AB016078). Total DNA from *R. erythropolis* A4 was used as a template. The amplified fragment (about 0.3 kb) was cloned in *Escherichia coli* into the *Sma*I site

of the *E. coli* vector pK18mobsacB [18]. The resulting construct pNH343 was isolated from *E. coli* cells and introduced into *R. erythropolis* A4 by electrotransformation using the previously described protocol [17]. The regions flanking the cloned parts of *nha1* and *nha2* were isolated by the plasmid-rescue technique from the clones with the plasmid pNH343 (selected on kanamycin) integrated into the *R. erythropolis* A4 chromosome. The sequences of the genes *nha1* and *nha2* and the flanking regions were determined by primer walking using the ABI Prism 2100 sequencer (Perkin-Elmer). The resulting nucleotide sequence (1701 bp) was deposited in GenBank database under accession no. AM710613.

## 3. Results and discussion

### 3.1. Biotransformations catalyzed by the semi-purified nitrile hydratase

When using whole cells of *R. erythropolis* A4, the end products of nitriles examined in this study were mostly carboxylic acids. In some cases, careful monitoring of the reaction course enabled to obtain amides of reasonable purity (unpublished results). However, a semi-purified nitrile hydratase used in this work proved to be a biocatalyst of choice for the synthesis of pure amides from nitriles. The enzyme was partially purified in two chromatographic steps [6] and applied to transformation of nitriles **1a–13a** (Tables 1–3). The enzyme was not very sensitive to steric hindrances imposed by branched and substituted nitriles. Only the most bulky nitriles **9a** and **13a** reacted sluggishly but the corresponding amides **9b** and **13b** (Table 2, entry 9 and Table 3, entry 13, respectively) could be obtained by either extending the reaction time and/or increasing enzyme/substrate ratio. 3-Oxonitriles **1a–4a** (Table 1) also required high enzyme/substrate ratio for total conversion. This was probably caused by deactivation of the enzyme in presence of compounds containing reactive oxo groups. The conversion of all compounds except for **9a** was nearly quantitative and the isolated yields of the corresponding amides were in general good to excellent.

The utility of the semi-purified enzyme was also demonstrated by the chemoselective transformation of cyano ester **13a**.

When ester groups were present in the substrate molecules, whole cells often gave deesterified products due to presence of esterases [1], which are ubiquitous in microorganisms. On the other hand, no ester hydrolysis was observed during the reaction catalyzed by the semi-purified nitrile hydratase (see Table 3, entry 13). However, the sterically hindered nitrile required high enzyme/substrate ratio and extended reaction time for full conversion.

### 3.2. Enantioselectivity of the nitrile hydratase

The nitrile hydratase from *R. erythropolis* exhibited a good enantioselectivity towards several nitriles like 2-(4-methoxyphenyl)propionitrile, 2-(6-methoxy-2-naphthyl)propionitrile [6], *trans*-*N*-(2-cyanocyclohexyl)benzamide and *trans*-*N*-(2-cyanocyclohexyl)-4-methylbenzenesulfonamide [19]. In this work, enantioselectivity of the nitrile hydratase was examined with substrates **6a**, **7a**, **9a** and **10a**, as chiral separations of these nitriles or the corresponding amides were feasible using HPLC. The low *E* (2.8) of the hydration of (*R,S*)-2-( $\alpha$ -hydroxybenzyl)propenenitrile (**7a**) was not surprising due to the distant position of the stereogenic carbon atom from the reactive cyano group. Previously, low *E*-values were reported for the hydrolysis of this compound by whole cells of *Rhodococcus* sp., while the hydration step was found to be non-selective [20]. Addition of cosolvents (5–20% (v/v) methanol or pristane) did not cause any significant change of enantioselectivity (data not shown). The enantioselectivity of the nitrile hydratase for nitrile **6a** was similar (*E*=2.7). The hydration of nitriles **9a** and **10a** was non-selective.

### 3.3. Biotransformations using cross-linked nitrile hydratase aggregates

Recently we observed a strong inhibitory effect of ammonium ions on amidase activity of cell-free extracts from *R. erythropolis* (5% residual activity in the presence of 800 M ammonium sulfate [7]). This finding suggested that addition of ammonium ions may improve the purity of amides formed by crude nitrile hydratase preparations, provided the nitrile hydratase remains active at concentrations, which suppress the amidase activity. The effect of ammonium ions on both enzymes was examined using a cell-free extract in the form of cross-linked enzyme aggregates (CLEAs).

Approximately 88% of the nitrile hydratase activity was recovered after immobilization of the cell-free extract in CLEAs, when using ammonium sulfate as precipitant. The use of other precipitants resulted in complete deactivation of the enzyme. Comparison of glutaraldehyde and dextran polyaldehyde as cross-linking agents showed that only the latter compound was suitable for nitrile hydratase, while the former one was detrimental to the enzyme activity (data not shown). Recently, a similar effect of glutaraldehyde was observed with nitrilase [10]. The nitrile hydratase in CLEAs was stabilized by immobilization. The immobilized enzyme retained 77%, 46% and 25% of its initial activity after 1, 4 and 10 h, respectively, while the cell-free extract was less stable with 66% and 27% of residual activity

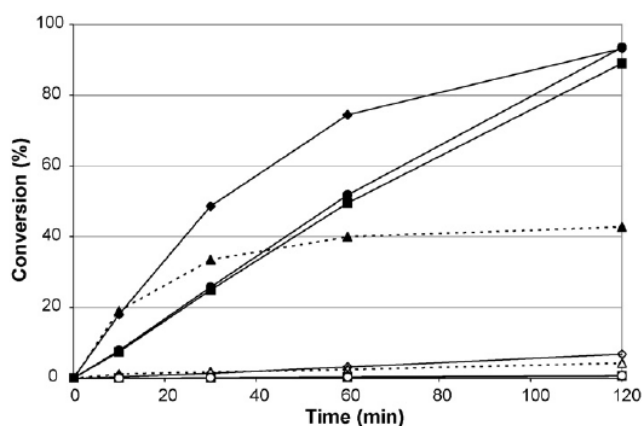


Fig. 1. Biotransformation of benzonitrile (25 mM) into benzamide (mM; closed symbols) and benzoic acid (mM; open symbols) by cell-free extract in absence of ammonium sulfate (triangles, broken line) and by cross-linked enzyme aggregates in absence (diamonds) or presence of 360 mM (circles) and 800 mM (squares) ammonium sulfate. The reaction mixture contained 0.5 U of nitrile hydratase mL<sup>-1</sup>. See Section 2 for details.

after 1 and 4 h, respectively. Comparison of benzonitrile conversion by cell-free extract and CLEAs also indicated a slower deactivation of the latter biocatalyst (see Fig. 1). Amidase activity was also present in CLEAs at approximately 77% of its initial activity. The selective inhibition of amidase was demonstrated in a CLEA-catalyzed conversion of benzonitrile (see Fig. 1). In absence of ammonium sulfate, the ratio of benzoic acid in the total product was approximately 6%. The addition of ammonium sulfate (360 or 800 mM) decreased this ratio by 8–10 times, the effect of both concentrations of the salt being similar. Furthermore, ammonium sulfate exhibited a stabilizing effect on the nitrile hydratase activity in CLEAs. In presence of any of the examined concentrations of ammonium sulfate, the reaction rate was almost stable during 120 min, while it slowed down after 30 min in the reaction mixture without ammonium sulfate. Thus, despite a lower initial reaction rate (by about one half), the biotransformations carried out in presence of ammonium sulfate afforded comparable benzamide yields as the control without ammonium sulfate.

Previously it was reported that whole cells of *R. rhodochrous* produced significant amounts of 3-oxo-3-phenylpropanoic acid (**1d**) together with acetophenone (product of decarboxylation of **1d**) from nitrile **1a**, unless the microorganism was grown in the presence of diethylphosphoramidate [4]. The same reaction pattern was observed for the CLEA-catalyzed reaction in buffer without ammonium sulfate (see Table 4, entry 14). However, in presence of 360 mM ammonium sulfate the ratio of amide in total product was 75% after full conversion of **1a** (see Table 4, entry 15). At 800 mM ammonium sulfate, the rate of nitrile hydration decreased and total conversion was not achieved after 180 min. On the other hand, the purity of the amide was the highest, total product consisting of 87% amide (see Table 4, entry 16). Similar effects of ammonium sulfate were observed for the transformation of (*R,S*)-2-(1-hydroxyethyl)propenenitrile (**5a**) by CLEAs (see Table 4, entries 17–19). The amide ratio in the total product

was 43% in absence of ammonium sulfate. At 360 mM ammonium sulfate acid formation was reduced by almost one half. The best purity of the amide was achieved at 800 mM, when the total product consisted of 89% amide. The above examples illustrated the utility of ammonium sulfate as an amidase inhibitor, which enabled to control the course of the reactions to give the desired products.

#### 3.4. Sequence analysis of the nitrile hydratase genes

Previously, we determined the N-terminal sequences of both subunits of the nitrile hydratase from *R. erythropolis* A4 [6] and stated that they were identical to those of the best-characterized nitrile hydratases from *Rhodococcus* sp. N-771 [21] and *Rhodococcus* sp. R 312 (formerly *Brevibacterium* sp. R 312) [22]. Therefore, different substrate specificities of the nitrile hydratase studied by us, on one hand, and those of strains N-771 and R 312, on the other hand, were surprising (regarding, in particular, a high activity of the enzyme from strain A4 towards aromatic nitriles [6]). To shed more light on the elutionary relationships between the nitrile hydratase from strain A4, which showed a very broad substrate specificity (as shown herein and previously, e.g. [6,15,19,23]), and the nitrile hydratases from other strains, the genes coding for  $\alpha$ - and  $\beta$ -subunits of nitrile hydratase were cloned and their nucleotide sequences determined. The DNA fragment (1286 nt) covering the genes *nha1* and *nha2* and their intergenic region (26 nt) from *R. erythropolis* A4 differs by 10 nt from the most similar homologous sequence from *R. erythropolis* 870-AN019 (GenBank accession no. AY223829) [24] according to the similarity search in GenBank database. The deduced *R. erythropolis* A4 polypeptides Nha1 ( $\alpha$ -subunit) and Nha2 ( $\beta$ -subunit) showed 100% aa identity with the deduced aa sequences of the nitrile hydratase subunits from three strains of *R. erythropolis* (67-BEN001, ANT-AN007 and ARG-AN025 [24] and with a sequence from the strain *R. globerulus* A-4 [25]. Some data on substrate specificity are available for strain 67-BEN001, which accepts aliphatic (di)nitriles, benzonitrile and 3-cyanopyridine [26].

All deduced aa sequences of other nitrile hydratases from *Rhodococcus* strains, whose substrate specificities were studied (strains N-774, N-771, AJ270 and R312), are highly similar to each other [24] and differ in the same six aa from *R. erythropolis* A4-deduced sequence. Considering the high degree of aa identity, similar substrate specificities of these enzymes could be expected. However, different substrate specificities of nitrile hydratases from *Rhodococcus* sp. (formerly *Brevibacterium* sp.) R312 [27] and from *R. erythropolis* A4 [6] have been described. The use of enzymes of a different purity or different experimental conditions could be responsible for the observed differences. Nevertheless, it is still possible that a few amino acid substitutions could modify the substrate specificity. Other hypothesis suggests that the primary structure of the enzyme affects its quaternary structure (e.g. dimers versus tetramers) [22]. In turn, quaternary structure of the enzyme could influence its catalytic properties. Reasons for the variations in the enzyme properties still deserve further investigation.

#### 4. Conclusions

We searched for biocatalysts suitable for the hydration of nitriles into the corresponding amides. The semi-purified nitrile hydratase from *R. erythropolis* A4 accepted a broad range of branched and substituted nitriles and proved to be utilizable for the preparative biotransformations of these compounds into amides as sole products. A benefit of this approach is the simple work-up of the reaction mixture containing very small amounts of biological material ( $<0.03$  mg of protein mL<sup>-1</sup>) and nearly no residual substrate or by-product. Therefore, extraction with an organic solvent was sufficient to isolate the amides in most cases. This method proved useful for enzymatic synthesis of several important types of amides known as versatile intermediates for a large number of natural compounds and products with pharmaceutical impact like 3-oxoamides [4], 3- or 4-hydroxylated 2-methyleneamides [13] and 3-hydroxyamides [28].

Cross-linking of aggregates of the crude nitrile hydratase with dextran polyaldehyde provided another enzyme formulation suitable for the transformation of nitriles into amides. Using this biocatalyst, amides could be obtained as major products, when the reaction proceeded in presence of a selective amidase inhibitor, ammonium sulfate (360–800 mM). Alternatively, the reaction conditions can be adjusted to favour carboxylic acid formation, as amidase also retained its activity in cross-linked enzyme aggregates.

Nitrile hydratases seem to be highly conserved in evolution. From the highly similar nitrile hydratases from rhodococci, the enzyme studied by us belongs to a few ones for that a large number of substrates was reported.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2007.09.007.

#### References

- [1] L. Martínková, V. Mylerová, *Curr. Org. Chem.* 7 (2003) 1279.

- [2] M. Kashiwagi, K.-I. Fuhshuku, T. Sugai, *J. Mol. Catal. B: Enzym.* 29 (2004) 249.
- [3] R. Bauer, B. Hirrlinger, N. Layh, A. Stolz, H.-J. Knackmuss, *Appl. Microbiol. Biotechnol.* 42 (1994) 1.
- [4] V. Gotor, R. Liz, A.M. Testera, *Tetrahedron* 60 (2004) 607.
- [5] M. Cantarella, L. Cantarella, A. Gallifuoco, A. Spera, *Enzyme Microb. Technol.* 38 (2006) 126.
- [6] I. Přepechalová, L. Martínková, A. Stolz, M. Ovesná, K. Bezouška, J. Kopecký, V. Křen, *Appl. Microbiol. Biotechnol.* 55 (2001) 150.
- [7] V. Vejvoda, O. Kaplan, D. Kubáč, V. Křen, L. Martínková, *Biocatal. Bio-transform.* 24 (2006) 414.
- [8] L.Q. Cao, F. van Rantwijk, R.A. Sheldon, *Org. Lett.* 2 (2000) 1361.
- [9] R. Schoevaart, M.W. Wolbers, M. Golubovic, M. Ottens, A.P.G. Kieboom, F. van Rantwijk, L.A.M. van der Wielen, R.A. Sheldon, *Biotechnol. Bioeng.* 87 (2004) 754.
- [10] C. Mateo, J.M. Palomo, L.M. van Langen, F. van Rantwijk, R.A. Sheldon, *Biotechnol. Bioeng.* 86 (2004) 273.
- [11] R.L. Rajender, R.K. Rama, *Org. Prep. Proced. Int.* 32 (2000) 185.
- [12] J.S. Hill, N.S. Isaac, *J. Chem. Res. Miniprint* 10 (1988) 2641.
- [13] D. Crestia, C. Guérard, H. Veschambre, L. Hecquet, C. Demuyneck, J. Bolte, *Tetrahedron: Asymmetry* 12 (2001) 869.
- [14] C.J. Eby, C.R. Hauser, *J. Am. Chem. Soc.* 79 (1957) 723.
- [15] V. Vejvoda, O. Šveda, O. Kaplan, V. Přikrylová, V. Elišáková, M. Himl, D. Kubáč, H. Pelantová, M. Kuzma, V. Křen, L. Martínková, *Biotechnol. Lett.* 29 (2007) 1119.
- [16] M.J. DiGeronimo, A.D. Antoine, *Appl. Environ. Microbiol.* 31 (1976) 900.
- [17] M. Veselý, M. Pátek, J. Nešvera, A. Čejková, J. Masák, V. Jirků, *Appl. Microbiol. Biotechnol.* 61 (2003) 523.
- [18] A. Schäfer, A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, A. Pühler, *Gene* 145 (1994) 69.
- [19] M. Winkler, L. Martínková, A.C. Knall, S. Krahulec, N. Klempier, *Tetrahedron* 61 (2005) 4249.
- [20] M.-X. Wang, Y. Wu, *Org. Biomol. Chem.* 1 (2003) 535.
- [21] M. Nakasako, M. Odaka, M. Yohda, N. Dohmae, K. Takio, N. Kamiya, I. Endo, *Biochemistry* 38 (1999) 9887.
- [22] W.J. Huang, J. Jia, J. Cummings, M. Nelson, G. Schneider, Y. Lindquist, *Structure* 5 (1997) 691.
- [23] L. Martínková, N. Klempier, M. Preiml, M. Ovesná, M. Kuzma, V. Mylerová, V. Křen, *Can. J. Chem.* 80 (2002) 724.
- [24] P.F.B. Brandao, J.P. Clapp, A.T. Bull, *Appl. Environ. Microbiol.* 69 (2003) 5754.
- [25] S.X. Xie, Y. Kato, Y. Asano, *Biosci. Biotechnol. Biochem.* 65 (2001) 2666.
- [26] S.C. Heald, P.F.B. Brandao, R. Hardacre, A.T. Bull, *Antonie van Leeuwenhoek* 80 (2001) 169.
- [27] T. Nagasawa, K. Ryuno, H. Yamada, *Biochem. Biophys. Res. Commun.* 139 (1986) 1305.
- [28] Z.-L. Wu, Z.-Y. Li, *J. Mol. Catal. B: Enzym.* 22 (2003) 105.