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Biotransformation of nitriles to hydroxamic acids via a nitrile hydratase–amidase cascade reaction

Vojtěch Vejvoda[a], Ludmila Martínková[a,*], Alicja B. Veselá[a], Ondřej Kaplan[a], Sabine Lutz-Wahl[b], Lutz Fischer[b], Bronislava Uhnáková[a]

[a] Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic
[b] University of Hohenheim, Institute of Food Science and Biotechnology, Department of Biotechnology, 70599 Stuttgart, Germany

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ABSTRACT

The transformation of benzonitrile into benzohydroxamic acid was performed by a cascade bienzymatic reaction involving nitrile hydration and acyl transfer of the intermediate benzamide onto hydroxylamine. The first step was catalyzed by either a cell-free extract from *Rhodococcus erythropolis* A4 (nitrile hydratase) or cell-free extracts from recombinant *Escherichia coli* strains expressing nitrile hydratases from *Raoultella terrigena* 77.1 and *Klebsiella oxytoca* 38.1.2; the biocatalyst in the second step was a cell-free extract from *R. erythropolis* A4 (amidase). When using the cell-free extract from *R. erythropolis* A4 in the first step, the hydrolytic amidase activity was suppressed by ammonium ions, which, however, did not inhibit the acyl transfer reaction catalyzed by the same enzyme. Aromatic and aliphatic nitriles were examined as substrates of the recombinant nitrile hydratases by using a colorimetric assay of hydroxamic acids produced in a coupled acyl transfer reaction.

1. Introduction

Amidases have been widely applied as biocatalysts in the hydrolysis of amides. With respect to the stability of the amide bond, which requires drastic conditions for hydrolysis, a mild alternative method is very attractive. Furthermore, a large number of amidases exhibited outstanding enantioselectivities, thus opening a straightforward path to valuable chiral carboxylic acids [1–3]. These amidases are designated as "GGSS signature amidases" or "enantioselective amidases" and are encoded by genes located in clusters with nitrile hydratase α- and β-subunit genes [4].

Beside amide hydrolysis, amidases have also been used for acyl transfer from amides onto nucleophiles other than water (hydroxylamine or hydrazine [5]). The ability to catalyze this reaction was reported for different amidases, which were evolutionarily distant from each other. An enantioselective amidase from *Rhodococcus* sp. R312 catalyzed acyl transfer from various aromatic and aliphatic amides onto hydroxylamine [4]. The same strain produced another amidase ("wide-spectrum amidase" [6]), which exhibited no amino acid sequence similarity to the "enantioselective amidase" and belonged to the nitrilase superfamily [7] together with an amidase from *Pseudomonas aeruginosa*. These enzymes also catalyzed the acyl transfer but showed a preference for short-chain amides [8,9]. Hydroxamic acids are valuable products with a chelating ability for metal ions and have a wide number of applications in pharmaceutics [4,9]. Another amidase from *Rhodococcus erythropolis* MP50 [10] catalyzed an enantioselective acyl transfer from 2-phenylpropionamide onto hydroxylamine giving *S*-2-phenylpropionohydroxamic acid, which was subsequently converted to *S*-1-phenylethylamine by Lossen rearrangement [11]. The acyl transfer from amides onto hydroxylamine is also useful for colorimetric enzyme assays, as hydroxamic acids can be readily detected and quantified after complexation with ferric ions. This reaction was used, e.g., for a rapid estimation of amidase activity from *R. erythropolis* with acetamide as the substrate [12] or screening of microorganisms with *R*-specific amidase activity for (*R*,*S*)-2,2-dimethyl cyclopropanecarboxamide [13].

The mild hydrolysis of nitriles into carboxylates has been widely performed using the bienzymatic pathway employing nitrile hydratase and amidase. Here we examined an analogous method, which made use of the same type of enzymes but a different acyl acceptor (hydroxylamine). The use of nitriles as starting material for hydroxamic acid production would be beneficial because of the ease of nitrile synthesis via a variety of reactions and their good commercial availability. This method is also applicable

* Corresponding author.
E-mail address: martinko@biomed.cas.cz (L. Martínková).

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to coupled nitrile hydratase assays, provided a suitable amidase is available for catalysis of the acyl transfer reaction.

2. Experimental

2.1. Chemicals

L- and D-Phenylglycinamide was kindly donated by DSM. Other amides and nitriles were purchased from standard commercial sources (Alfa Aesar, Aldrich, Fluka) and were of the highest purity available (≥95%).

2.2. Bacterial strains and cultivation

R. erythropolis A4 (deposited in the Culture Collection of Microorganisms, Masaryk University Brno) was grown as described previously [14].

The cultivation of the recombinant strains expressing nitrile hydratases from *Klebsiella oxytoca* 38.1.2 and *Raoultella terrigena* 77.1 was performed as described previously [15]. The construction of the expression plasmids was performed as described in Section 2.3. Freshly transformed cells from *Escherichia coli* BL21(DE3) pET22_38.1.2a/pET26_38.1.2b and *E. coli* BL21(DE3) pET22_77.1a/pET26_77.1b were grown overnight at 37 °C in 5 mL LB-medium containing 100 μg mL^{-1} ampicillin, 25 μg mL^{-1} kanamycin and 2% (w/v) glucose. 5 mL of this culture was used to inoculate 500 mL of LB-medium containing 100 μg mL^{-1} ampicillin, 25 μg mL^{-1} kanamycin and 1% (w/v) glucose and incubated at 30 °C and 160 rpm. At an optical density OD_{600} = 1 the incubation temperature was dropped down to 20 °C and the cells were induced by addition of 0.4 mM IPTG and 250 μM $CoCl_2 \cdot 6H_2O$. The cells were harvested 24 h after induction.

2.3. Construction of expression plasmids

The genes of the α- and β-subunits of the nitrile hydratases from *K. oxytoca* 38.1.2 or *R. terrigena* 77.1 were amplified by PCR from the genomic DNA. Primers used were designed according to the nucleotide sequence of the genes [15] which introduced an additional *Nde*I restriction site at the N-terminal sequence and a *Xho*I site just behind the stop codon. After digestion with the *Nde*I and *Xho*I, the fragments were inserted in the expression vectors pET22b (α-subunits) and pET26b (β-subunits) respectively.

2.4. Preparation of cell-free extracts

Cell-free extracts of *R. erythropolis* A4 were prepared as described previously [16] using a Retsch MM-200 oscillation mill (under slightly modified conditions: 30 Hz, 30 min).

To prepare cell-free extracts of the recombinant *E. coli* strains the cells were suspended in Tris/HCl buffer (100 mM, pH 7.0) containing 43.5% (w/v) glycerol and disrupted by sonification. Cell debris was removed by centrifugation.

2.5. Determination of protein

Protein was determined according to Bradford [17] using bovine serum albumin as the standard.

2.6. Enzyme assays

Amidase activity was determined in reaction mixtures (1 mL) consisting of Tris/HCl buffer (50 mM, pH 8), 15 mM benzamide (from a 250 mM stock solution in methanol) and an appropriate amount of cell-free extract from *R. erythropolis* A4. After 5-min pre-incubation at 45 °C under shaking, the reaction was started by substrate addition, carried out for 10 min under the same conditions and quenched with 200 μL of 1 M HCl. The precipitate was removed by centrifugation (14,000 × *g*, 5 min). Nitrile hydratase activity was determined in an analogous way, using 25 mM benzonitrile as the substrate at 35 °C. Hydroxylamine was added at concentrations of 10–100 mM to test the effect of this compound on nitrile hydratase activity.

The acyl transferase activity of the amidase was determined at 28 °C. The reaction mixture (total volume 1 mL) consisted of 500 mM hydroxylamine (from a 2 M aqueous stock solution prepared daily and neutralized with 40 μL 10 M NaOH mL^{-1}), 15 mM of benzamide (from a 500 mM stock solution in methanol) and an appropriate amount of cell-free extract in Tris/HCl buffer (50 mM, pH 8). Ammonium sulfate was added at concentrations of 50–800 mM to test the effect of this compound on acyl transferase activity. After 30 min, the reaction mixtures were analyzed by HPLC (see below).

The enzyme activity was defined as the amount of enzyme required either for the production of 1 μmol of benzamide (nitrile hydratase), benzoic acid or benzohydroxamic acid (amidase) per min under the conditions mentioned above.

2.7. Determination of optimum amide and hydroxylamine concentrations

The acyl transfer reactions were performed as described above but using differing amounts of benzamide (2.5–25 mM) and hydroxylamine (50–500 mM).

2.8. Determination of substrate specificity

To determine the acyl transferase substrate specificity, 15 mM of various amides (from a 500 mM stock solution in methanol) were used under the same conditions as described for enzyme assays but the reaction time was prolonged to 200 min. The reaction mixtures were analyzed spectrophotometrically (see below).

2.9. General procedure for bienzymatic conversion of nitriles into hydroxamic acids

A cell-free extract from *R. erythropolis* A4 or recombinant nitrile hydratases from strains 38.1.2 and 77.1 were used as catalysts in the first reaction step (nitrile hydration), which proceeded at 20 °C and pH 8 in Tris/HCl buffer (50 mM, pH 8) containing 15 mM substrate, methanol (3%, v/v) as cosolvent, and an appropriate amount of the nitrile hydratase. Ammonium sulfate was added to a final concentration of 240 mM when using the cell-free extract from strain A4. After 48 h, hydroxylamine (from a 2 M stock solution) was added to a final concentration of 500 mM, the temperature adjusted to 28 °C, and the reaction started by adding another portion of cell-free extract. The reaction was monitored spectrophotometrically over 200 min (see below).

2.10. Spectrophotometric determination of hydroxamic acids

A 125 μL aliquot of the reaction mixture was added to 25 μL of 1 M HCl and centrifuged at 10,000 × *g* for 5 min. 100 μL of the supernatant was mixed with 100 μL of $FeCl_3$ solution (365 mM in 0.65 M HCl) in a 96-well polystyrene plate and absorbance was measured at 492 nm. Calibration of the assay was performed with 0–1 mM benzohydroxamic acid.

2.11. Analytical HPLC

The concentrations of benzonitrile, benzamide, benzoic acid and benzohydroxamic acid were determined using an HPLC configu-

Table 1
Substrate specificity of amidase from *Rhodococcus erythropolis* A4 for acyl transferase activity.

Substrate	Response level with amidase	Response of spontaneous non-enzymatic reaction
Benzamide	+++	–
3-Toluamide	+++	–
4-Toluamide	++	–
2-Chlorobenzamide	++	–
4-Chlorobenzamide	+	–
3-Aminobenzamide	++	–
4-Aminobenzamide	+	–
Nicotinamide	–	–
Isonicotinamide	–	–
Propionamide	+++	–
Butyramide	+++	–
Valeramide	+++	–
Cyclohexancarboxamide	+++	–
Methacrylamide	++	+
L-Phenylglycinamide	–	–
D-Phenylglycinamide	–	–
D,L-Lactamide	–	++

The amides (15 mM each) were incubated with the cell-free extract from *R. erythropolis* A4 and hydroxylamine (500 mM) in Tris/HCl buffer (50 mM, pH 8) at 28 °C for 200 min. Amidase were omitted from the blanks.
–, +, ++, +++ represents the intensity of absorbance at 492 nm after mixing the sample with acidic $FeCl_3$ (– < 0.15; + 0.15 – 0.4; ++ 0.4 – 0.7; +++ > 0.7).

ration from Thermo Separation Products (P4000 pump, AS 1000 autosampler, UV 2000 detector and SN 4000 degasser) and a GROMSIL 120 ODS 3CP column (125 mm × 4.6 mm, 3 μm particle size; Germany) with a mobile phase consisting of 50% acetonitrile, 49.7% water and 0.3% H_3PO_4 at a flow rate of 0.7 mL min^{-1}.

2.12. Extraction and purification of benzohydroxamic acid

The reaction mixture (15 mL) was centrifuged (10,000 × *g*, 20 min) and extracted with ethyl acetate (3 × 10 mL). The ethyl acetate fractions were pooled, evaporated at reduced pressure and the solid residue was dissolved in methanol. Purification proceeded on HPLC using a NovaPak HR C18 column (6 μm; 7.8 mm × 300 mm) and an aqueous mobile phase containing 1.5% acetonitrile at a flow rate of 4 mL min^{-1}.

3. Results and discussion

3.1. Nitrile hydratase and acyl transferase activities of R. erythropolis A4

Nitrile hydratases and enantioselective amidases are expressed simultaneously in a number of bacterial strains, including *R. erythropolis* A4. The substrate specificities of both enzymes are broad in this organism [2,14,18]. The nitrile hydratase–amidase operon of *R. erythropolis* A4 was recently sequenced [14]. The amino acid identities of the nitrile hydratase and amidase to those from *Rhodococcus* sp. R312 were 99% and 96% respectively, but the range of substrates accepted by the nitrile hydratase of the A4 strain seemed to be broader [14].

The cell-free extract from *R. erythropolis* A4, as a readily available source of nitrile hydratase and amidase (approximately 4 and 1 U mg^{-1} protein at 35 and 45 °C, respectively), was a prospective biocatalyst for both reaction steps required for the synthesis of hydroxamic acids from nitriles. The acyl transferase activity of the amidase was examined with benzamide. In the control reaction without enzyme, there was no response to this substrate, unlike some other amides (see Table 1). The spectrophotometric assay indicated that the amidase from *R. erythropolis* A4 catalyzed the acyl transfer from benzamide onto hydroxylamine. Using an authentic standard of benzohydroxamic acid, formation of this compound in the reaction mixture was confirmed by HPLC. The rates of acyl transfer from 15 mM benzamide increased with increasing concentrations of hydroxylamine (50–500 mM), the highest ones being observed at ≥250 mM. Those at 50–100 mM hydroxylamine still exceeded 90% of the maximum reaction rate. Contrary, the ratio of benzoic acid in the reaction product decreased with increasing hydroxylamine concentrations (from 18% at 50 mM hydroxylamine to 13, 8 and 5% at 100, 250 and 500 mM respectively). This was in accordance with a previous optimization of hydroxylamine concentrations in benzohydroxamic acid production, where 500 mM concentration was also the best one [4]. When using 500 mM hydroxylamine, the reaction rate decreased at benzamide concentrations below 10 mM (to 86 and 56% at 5 and 2.5 mM respectively). Therefore, the reaction was performed with 15 mM benzamide and 500 mM hydroxylamine. Under these conditions, the acyl transferase activity determined at 28 °C (due to volatility of hydroxylamine) was approximately 0.6 U mg^{-1} protein.

3.2. Substrate specificity of the acyltranferase in R. erythropolis A4

The amidase from the A4 strain hydrolyzed a wide range of structurally diverse amides [2]. Hence, we expected that this enzyme would also have a broad substrate specificity in acyl transfer reactions. The biotransformations of various aliphatic and aromatic amides by the cell-free extract from this bacterium confirmed this assumption (see Table 1). The reactions catalyzed by the amidase from the A4 strain gave comparable response levels with benzamide and aliphatic amides (propionamide, butyramide, valeramide), whereas the amidase from the R312 strain [4] preferred the latter substrates. The enzyme from strain A4 was also active with a number of aromatic amides (toluamides, chlorobenzamides, aminobenzamides) not examined with strain R312. On the other hand (iso)nicotinamide and lactamide seemed to be only transformed by the enzyme from the R312 strain.

The assay was calibrated for benzohydroxamic acid. High response levels (OD_{492} > 1) obtained in the reactions of benzamide were outside the linear range of the assay, indicating presence of ≥2 mM benzohydroxamic acid in the reaction mixture. In addition, absorption coefficients for Fe(III) complexes of propionohydroxamic, butyrohydroxamic and valerohydroxamic acid were calculated previously (1029, 1016 and 1023 L mol^{-1} cm^{-1} respectively) [4]. By using these coefficients, which were determined for a similar wavelength (500 nm), the concentration of propionohydroxamic, butyrohydroxamic or valerohydroxamic acid produced from the corresponding amides (Table 1) was calculated to be ≥1.6 mM (more accurate determination being not possible either due to too high response levels).

3.3. Benzohydroxamic acid production from benzonitrile by cell-free extract from R. erythropolis A4

Using benzonitrile as the substrate, we examined the compatibility of the nitrile hydration and acyl transfer reactions catalyzed by the cell-free extract from *R. erythropolis* A4. Nearly all the nitrile hydratase activity for benzonitrile was lost in presence of ≥10 mM of hydroxylamine. Therefore, the transformation of benzonitrile into benzohydroxamic acid could not be carried out as a one-pot synthesis.

We also observed an adverse effect of hydroxylamine on the amidase, which was totally inactivated after a 30 min preincubation with 500 mM hydroxylamine. However, the enzyme was much more stable in the reaction mixture containing the same concentration of hydroxylamine and 15 mM benzamide, where it retained 80% of its initial activity after the same period. This observation indicated that the substrate had a stabilizing effect.

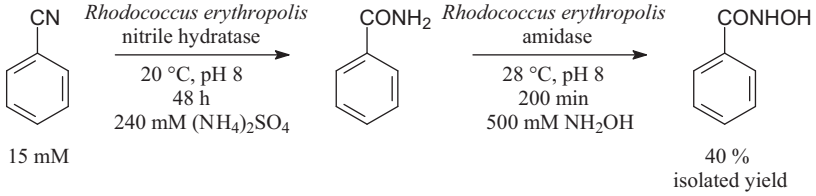

Fig. 1. Enzymatic synthesis of benzohydroxamic acid from benzonitrile using *Rhodococcus erythropolis* A4 cell-free extract with nitrile hydratase and amidase activities.

When using the cell-free extract as the catalyst of the nitrile hydration step, the hydrolytic activity of the amidase resulted in the formation of benzoic acid. Therefore, we searched for reaction conditions that would restrict benzamide hydrolysis. Recently, we observed that the activity of this amidase was reduced in the presence of ammonium ions [14]. Ammonium sulfate concentrations of 40, 80, 160 and 240 mM lowered the hydrolytic activity of the amidase to 33, 25, 17 and 15% of the control, respectively, but increasing this concentration up to 720 mM was not too efficient, the residual activity not decreasing below 10%. Hence, a medium concentration of ammonium sulfate (240 mM) was chosen, which enabled to transform 15 mM benzonitrile into an almost pure product consisting of ≥95% benzamide.

We subsequently examined if the reaction mixture after the hydration step could be used for the acyl transfer step. We assumed that the apparent inhibition of amidase by ammonium ions was caused by competition between water and ammonia (a stronger nucleophile) as acyl acceptors. If this were the case, the acyl transfer onto hydroxylamine (stronger nucleophile than ammonia) would proceed even in the presence of ammonium sulfate. This expectation was confirmed by the observation that the amount of acylated hydroxylamine was almost the same in absence of ammonium sulfate and its presence (over the whole tested range of 50–800 mM). The decrease in reaction rate did not exceed 10% at the highest concentrations. Neither had the presence of ammonia any significant effect on the ratio of benzohydroxamic and benzoic acid produced.

Hence, the reaction mixture from the first step was directly used for the acyl transfer reaction. This reaction was started by adding hydroxylamine and another portion of the cell-free extract, the amidase activity added in the first portion being largely lost after the first step. In this way, the total conversion of 15 mM benzonitrile was achieved to give benzohydroxamic acid (isolated yield 40%, purity ≥95%; Fig. 1), the structure of which was confirmed by ^{1}H and ^{13}C NMR spectra (see Supplementary data).

3.4. Benzohydroxamic acid production from benzonitrile by recombinant nitrile hydratases and the amidase from R. erythropolis A4

Application of a nitrile hydratase catalyst that is devoid of amidase activity would make the use of ammonium salt in the first step unnecessary. To this end, the cell-free extracts from *E. coli* cells expressing recombinant nitrile hydratases were tested. The specific activities of these extracts (0.09 and 0.65 U mg^{-1} protein for strains 77.1 and 38.1.2, respectively) were lower than that of the extract from the A4 strain. However, the lack of amidase activity in *E. coli* cells was an advantage.

The nitrile hydratases from strains 77.1 and 38.1.2 retained approximately one fifth of their initial activity in 100 mM hydroxylamine and thus seemed to be more resistant towards this compound than the enzyme from the A4 strain. This can be attributed to the fact that the former enzymes belong to Co-type nitrile hydratases, which are often more stable than Fe-type nitrile hydratases at elevated temperatures or in the presence of organic solvents [2,19]. Glycerol, in which the recombinant enzymes were stored, could also play a protective role. Nevertheless, their use at the higher hydroxylamine concentrations required for the acyl transfer was not possible either. Therefore, the hydration of benzonitrile (15 mM) was performed in the absence of hydroxylamine. Benzamide was then converted into almost pure benzohydroxamic acid by the cell-free extract from the A4 strain.

Table 2
Substrate specificity of recombinant nitrile hydratases determined spectrophotometrically after coupling with enzymatic acyl transfer.

Substrate	Response level with nitrile hydratase from		Response of control
	Strain 38.1.2	Strain 77.1	
Benzonitrile	++	+	–
3-Tolunitrile	++	+	–
4-Tolunitrile	–	–	+
3-Chlorobenzonitrile	++	–	–
4-Chlorobenzonitrile	+	+	–
3-Hydroxybenzonitrile	+	+++	–
4-Aminobenzonitrile	+	+	–
Propionitrile	++	++	+
Butyronitrile	+++	++	+
Valeronitrile	+++	++	+
2-Methyl-3-butenenitrile	++	+	+

The nitriles (15 mM each) were incubated with a recombinant nitrile hydratase at room temperature for 48 h. Afterwards, hydroxylamine (500 mM) and an appropriate amount of the cell-free extract from *R. erythropolis* A4 were added and the acyl transfer reaction performed in Tris/HCl buffer (50 mM, pH 8) for 200 min at 28 °C. Amidase were omitted from the blanks.
–, +, ++, +++ represents the intensity of absorbance at 492 nm after mixing the sample with acidic $FeCl_3$ (– < 0.15; + 0.15 – 0.4; ++ 0.4 – 0.7; +++ > 0.7).

3.5. Screening the nitrile hydratase substrate specificity

Bienzymatic conversion of nitriles into hydroxamic acids was also used to screen for the substrates of recombinant nitrile hydratases from strains 77.1 and 38.1.2 (see Table 2). The amidase from *R. erythropolis* A4 showed a broad substrate specificity in acyl transfer reactions (see above) and thus seemed to be suitable for the detection of a wide range of amides resulting from the action of nitrile hydratases. When comparing the responses from different nitriles, however, the differing relative rates of acyl transfer reaction (Table 1) must be considered as well as the responses of the spontaneous non-enzymatic reaction (see Tables 1 and 2). In some cases, positive response of the control (without enzyme) occurred with nitriles (propio-, butyro-, valeronitrile) but not with the corresponding amides. This could not be accounted to spontaneous reactions, as no amide was present in the reaction mixture. Instead, this response was attributed to interference of the reagent with the nitrile compound or impurity present in it. Response levels from hydroxamic acid complexes with known absorbance coefficients were used to calculate the concentrations of the corresponding hydroxamic acids (approximately 1.3 mM benzohydroxamic acid, 0.95 mM propionohydroxamic acid and ≥2.5 mM butyrohydroxamic or valerohydroxamic acid, the responses from the latter two compounds being off the linear range of the assay). However, these concentrations need not cor-

respond to the concentration of amides as these intermediates could be only partly converted into hydroxamic acids. Hence, the assay must be taken as qualitative to semiquantitative. Moreover, calibration for other hydroxamic acids was not possible in this study as standards or absorption coefficients were missing. Nevertheless, the screening clearly showed the activity of both nitrile hydratases for both aromatic and aliphatic substrates, i.e., benzonitrile, 3-tolunitrile, 3-hydroxybenzonitrile, 4-chlorobenzonitrile, 4-aminobenzonitrile, propionitrile, butyronitrile and valeronitrile. The assay also revealed that the conversion of almost all substrates except for 3-hydroxybenzonitrile was higher in strain 38.1.2 than in strain 77.1. 3-Chlorobenzonitrile and 2-methyl-3-butenenitrile seemed to be only converted by the former strain.

The assay was not useful for 3- or 4-cyanopyridine because it was impossible to detect any acyl transfer reaction of the corresponding amides. Obviously, the acyl transferase activity of the amidase was too low for these substrates, although the hydrolytic activity of the same enzyme for isonicotinamide was remarkable and could be utilized for the conversion of this compound into isonicotinic acid [16].

A number of colorimetric ammonia assays (see [20] for review, [21]) became available to monitor the activity of amidase or nitrilase. In principle, these methods were also utilizable for the determination of nitrile hydratase activity if a suitable enzyme (amidase) was available for use in the coupled reaction – transformation of amide into ammonia and carboxylic acid. The main drawback of this method seemed to be its possible interference with formation or consumption of ammonia in other reactions proceeding in crude-enzyme samples [22]. Contrary, the method used here was specific for the product of the amidase-catalyzed reaction if no interference occured. Substrates which fulfilled this requirement (most of the aromatic nitriles or amides tested here) were suitable for this assay.

4. Conclusions

The bienzymatic nitrile transformation into the corresponding hydroxamic acid was demonstrated with aromatic and aliphatic nitriles as substrates and catalysts (nitrile hydratases, amidase) from various sources. The preparative-scale biotransformation of benzonitrile into benzohydroxamic acid did not require isolation of the intermediate benzamide and was optimized to reduce the hydrolytic by-product benzoic acid. Analogous reactions will probably be applicable in the synthesis of other hydroxamic acids from, e.g., propionitrile, butyronitrile, valeronitrile or 3-tolunitrile, these products being detected colorimetrically. A colorimetric method based on the same bienzymatic reaction was used for a nitrile hydratase assay, which was suitable for the 96-well microtitration plate format. An amidase able to catalyze the acyl transfer from a variety of amides onto hydroxylamine is required for this method. Such an enzyme has been obtained from *R. erythropolis* A4, but other enzymes complementing its substrate specificity would be useful to extend the applicability of the assay. The qualitative or semiquantitive character of the results was outweighed by the rapidity and facility of the assay, which provided information on substrate specificity of new nitrile hydratases for a wide range of substrates. To make the assay more accurate, the protocol must be refined for each substrate and enzyme by finding suitable reaction conditions (for full reaction of amide intermediate) and appropriate sample dilution (to fit the absorbance values into the linear range of the color response).

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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.2011.03.008.

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