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Characterisation of nitrilase and nitrile hydratase biocatalytic systems

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Abstract Biocatalytic transformations converting aromatic and arylaliphatic nitriles into the analogous related amide or acid were investigated. These studies included synthesis of the β-substituted nitrile 3-hydroxy-3-phenylpropionitrile, subsequent enrichment and isolation on this substrate of nitrile-degrading microorganisms from the environment, and a comparative study of enzymatic reactions of nitriles by resting cell cultures and enzymes. Each biocatalyst exhibited a distinctive substrate selectivity profile, generally related to the length of the aliphatic chain of the arylaliphatic nitrile and the position of substituents on the aromatic ring or aliphatic chain. Cell-free nitrilases generally exhibited a narrower substrate range than resting whole cells of *Rhodococcus* strains. The *Rhodococcus* strains all exhibited nitrile hydratase activity and converted β-hydroxy nitriles (but did not demonstrate enantioselectivity on this substrate). The biocatalysts also mediated the synthesis of a range of α-hydroxy carboxylic acids or amides from aldehydes in the presence of cyanide. The use of an amidase inhibitor permits halting the nitrile hydratase/amidase reaction at the amide intermediate.

Introduction

Nitriles, amides, and carboxylic acids (particularly pure enantiomers of chiral compounds) have wide application in the pharmaceutical and chemicals industry. In synthesis, addition of a nitrile group achieves the addition of a carbon atom, while subsequent hydrolysis to the corresponding amide or carboxylic acid is of wide synthetic value. This hydrolysis may be achieved by a number of means, including application of biocatalysts (Banerjee et al. 2002). Nitrile-hydrolysing biocatalysis is due to the activity of one of two enzyme systems, nitrilase (EC 3.5.5.1), or nitrile hydratase (4.2.1.84) plus amidase (3.5.1.4) (acylamidase, acylase) (Scheme 1).

However, the scarcity of suitable and well-characterised nitrile-converting biocatalysts is a barrier to their application (DeSantis et al. 2002). Nitrile-converting enzymes are generally not commercially available (Martínková and Krěn 2002), are currently limited in variety (and therefore catalytic application), are usually labile, and are prone to product or substrate inhibition. Due to substrate insolubility in water, low conversion rates may also be a problem. Finally, much of the observed enantioselectivity in nitrile biocatalysis has been achieved through the hydrolysis of intermediate carboxamides by an amidase (in the nitrile hydratase pathway), yielding mostly the (*S*)-carboxylic acid (Faber 1992).

In spite of these limitations, there have been some commercial applications of nitrile biocatalysts. Prominent examples are the large-scale production of acrylamide (Mitsubishi Rayon Co.) and nicotinamide (Lonza AG) using a nitrile-hydratase-containing biocatalyst (*Rhodococcus rhodochrous*) (Wieser and Nagasawa 2000). Other compounds commercially produced using nitrilase biocatalysts are (*R*)-mandelic acid and (*R*)-3-chloromandelic acid (Mitsubishi Rayon Co). Although few commercial processes that utilise nitrilase and nitrile hydratase exist, there are a great many opportunities for their industrial application (Martínková and Krěn 2002), and hence many companies (such as DuPont, Lonza, Dow, Diversa, BASF, and DSM) have been investigating the application of nitrile biocatalysis for other processes. One application is the selective hydrolysis of nitriles in the presence of labile functional groups, which may not be possible using traditional strong acid or alkaline conditions (Sugai et al. 1997; Klempier et al. 1991).

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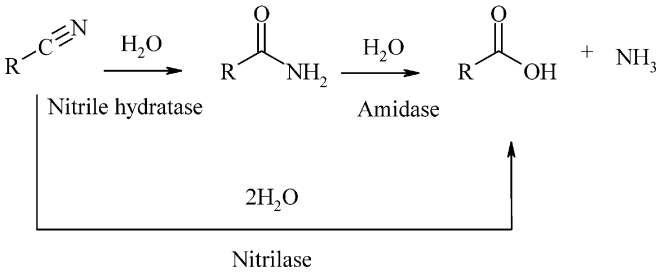


Scheme 1 Enzymatic hydrolysis of nitriles

Recent results have indicated that some nitrile-degrading enzymes can mediate kinetic resolutions through enantioselective hydrolysis of racemic nitriles. A purified cobalt-containing nitrile hydratase from *Pseudomonas putida* NRRL-18668 was stereospecific for 2(*S*)-(4′-chorophenyl)-3-methylbutyronitrile (Payne et al. 1997), which was hydrolysed 50 times faster than the (*R*)-enantiomer. Martínková et al. (1996) and Prepechalová et al. (2001) also observed stereoselective hydrolysis of 2-(4-methoxyphenyl)propionitrile by the nitrile hydratase system of *Rhodococcus equi* A4.

Nitrile-degrading enzymes may also be regioselective, preferring a single nitrile group in a dinitrile compound. For example, *Rhodococcus* AJ270, which hydrolyses a wide range of nitriles, dinitriles, and amides (Colby et al. 2000; Blakey et al. 1995), was found to be regiospecific for aliphatic dinitriles. Nitrilases can also discriminate between configurational isomers (*E*,*Z*) (Effenberger and Osswald 2001b). Additionally, they can be used in the synthesis of lactones from 4-hydroxy nitriles (Taylor et al. 1996), and lactams from α,ω-dinitriles (Gavagan et al. 1998)

Based on their versatility nitrile-converting enzymes are potentially widely applicable in organic synthesis. The present research was aimed at expanding the knowledge of the substrate profiles of a set of nitrile-converting biocatalysts.

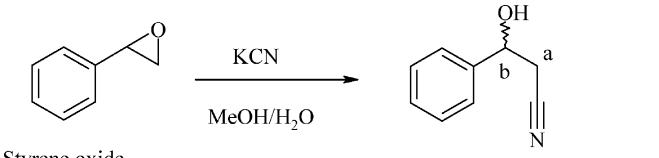
Materials and methods

Preparation of substrates

Preparation of (±)-3-hydroxy-3-phenylpropionitrile

β-Hydroxynitriles are of interest as intermediates in the synthesis of protease inhibitors for viruses. β-Hydroxynitriles are prone to elimination reactions, and therefore the mild hydrolysis conditions possible through biotransformation would be of value.

(±)-3-Hydroxy-3-phenylpropionitrile was prepared for this study (Scheme 2) by addition of potassium cyanide (5.43 g, 83.4 mmol) to a solution of methanol (50 ml) and water (20 ml) in a 250-ml round-bottomed flask fitted with a pressure-equalizing dropping funnel. To this homogeneous solution was added (±)-styrene oxide (2.03 g, 16.9 mmol) as a solution in methanol (15 ml) in a drop-wise fashion, affording a slightly murky solution. This was stirred for 18 h at room temperature, after which aqueous 1 M hydrochloric acid (80 ml) was added with spontaneous heating of the reaction. All gas evolved was scrubbed through a saturated aqueous sodium hydroxide solution. After 30 min of stirring, 70 ml of diethyl ether was added, and the organic phase isolated. This was washed with saturated aqueous



Styrene oxide

3-hydroxy-3-phenylpropionitrile

Scheme 2 Synthesis of β-substituted phenylpropionitrile

sodium hydrogen carbonate (2×50 ml), phase dried ($MgSO_4$), and concentrated to a viscous yellow oil. Flash chromatography [1:2 (v/v) ethyl acetate:hexane as eluent] afforded (±)-3-hydroxy-3-phenylpropionitrile (1.45 g, 59%); R_f 0.46 [1:2 (v/v) ethyl acetate: hexane]. Kamal and Khanna (2001) have recently published a similar preparation for racemic β-hydroxy compounds.

Preparation of aryl substituted (±)-2-amino-phenylacetonitriles

α-Hydroxy or α-aminonitriles are generated by adding an aldehyde and HCN to an aqueous buffered solution or aqueous ammonium chloride (Strecker synthesis, Scheme 3), respectively (Faber 1992; Vogel 1989; Weiner and Chaplin 2000).

Other nitrile compounds

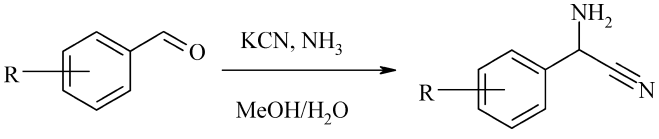
Many nitrile substrates were available from the laboratory compound inventory. All other substrates were obtained from Acros or Sigma.

Analytical methods

A Chromolith (Merck) speedrod (RP18e) C_{18} column eluted isocratically by 20% acetonitrile, 80% H_2O, 0.1% trifluroacetic acid (TFA) was used routinely for these experiments. The elution of aromatic compounds was monitored at 215 nm in all HPLC methods.

Chiral HPLC separations

Chiracel OD-RH, OD and OB-H columns, and Crownpak CR(+) (all from Daicel Chemical Industries, Japan) were used for chiral analysis. Chiracel OD-RH was used for the general resolution of α-hydroxy acids. Analytical conditions were: eluent 20% acetonitrile, 80% H_2O, 50 mM KH_2PO_4 buffer (adjusted with H_3PO_4 to pH 2). The flow rate was 0.5 ml /min. The Crownpak CR(+) was used to follow the hydrolysis of (*R*,*S*)-phenylglycinonitrile (Wegman et al. 2000, 2001). Analytical conditions were: perchloric acid ($HClO_4$) eluent at pH 2, 0.6 ml/min flow rate, Waters 625 LC pump, and a Waters 486 UV detector. The Chiracel OD column (Daicel, Japan) was used for determination of methyl ester derivatives of mandelic acid, and Chiracel OB-H was used to determine the levels of enantiomers of various carboxylic acid compounds. Both were eluted using mixtures of hexane and isopropanol.



R = H, Cl, F, OH, NO_2, CH_3

Scheme 3 Strecker synthesis of aryl substituted α-amino phenylaceronitriles

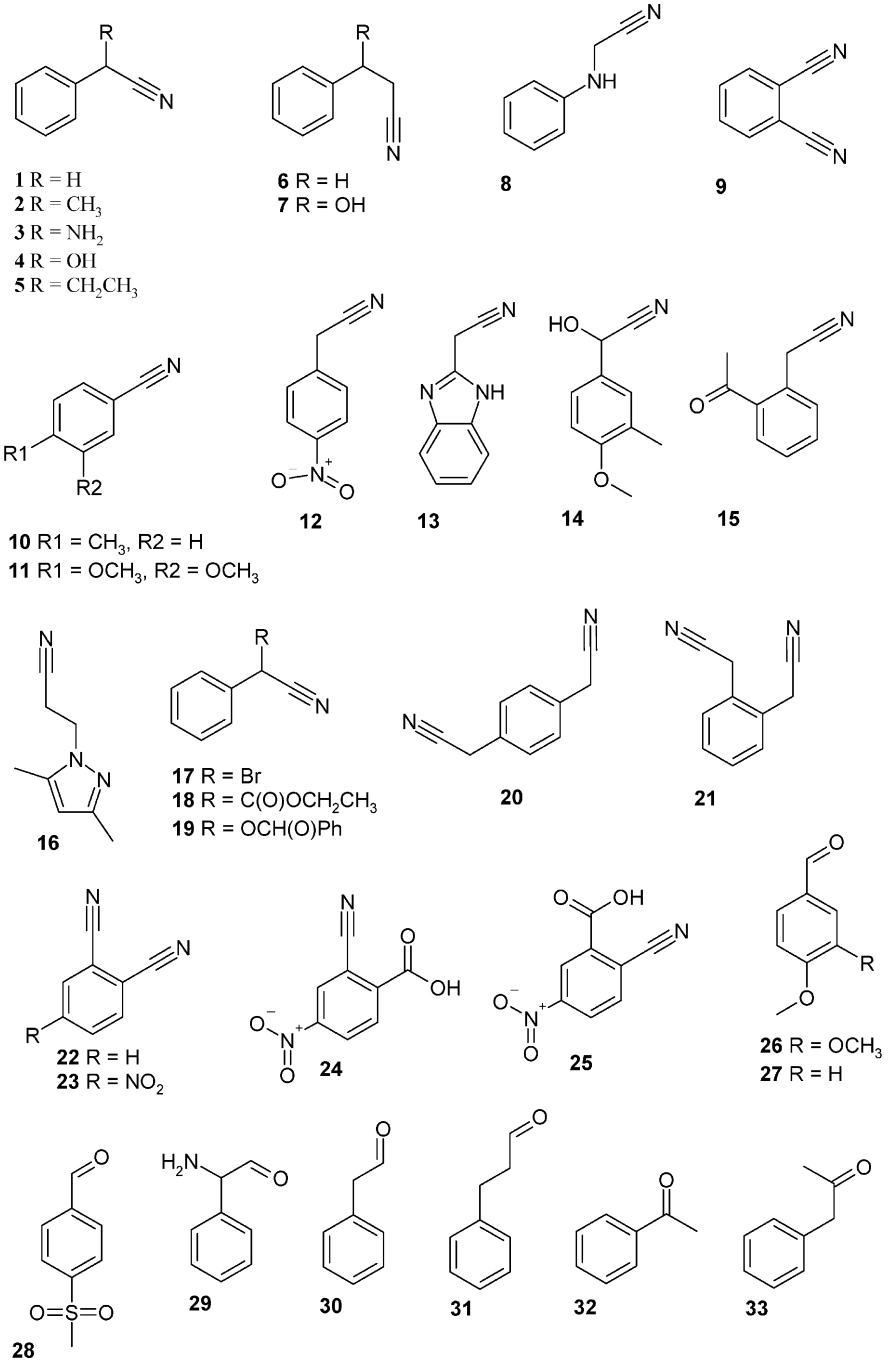
Microbial isolates

Nitrile-degrading microorganisms were isolated from the soil using the enrichment process derived from Layh et al. (1997) with an additional culturing on nutrient agar prior to the transfer to defined-medium agar plates containing 3-hydroxy-3-phenylproprionitrile as the nitrogen source. Isolates were grown in a liquid defined medium with 3-hydroxy-3-phenylproprionitrile, and preserved by lyophilization in the presence of milk powder as a stabiliser.

The organisms were characterised, with few exceptions, as species from the genera *Pseudomonas*, *Bacillus*, and *Rhodococcus* based on morphological and substrate-usage profiling. Biomass for resting cell experiments was obtained by growing cultures in a defined salt medium containing nitrile compounds (0.75 mM) as the sole nitrogen source, glucose (10 mM) as the carbon source, trace elements (Layh et al. 1997), and a vitamin solution. The vitamin solution was composed of (in g/l): biotin 0.05 (in 10 ml 0.1 mol/l NaOH, adjust to pH 6.5 with HCl), calcium pantothenate (1.0), nicotinic acid (1.0), myoinositol (25.0), thiamine HCl (1.0), *p*-aminobenzoic acid (0.2), pyridoxyl hydrochloride (1.0), adjusted to pH 6.5, and filter sterilised. Culture broth was centrifuged (5,000 g for 5 min), re-suspended in a small volume of phosphate buffer, and centrifuged again after dividing into aliquots in Eppendorf snaplock vials. Vials were rapidly frozen in liquid nitrogen and stored at −80°C, where they remained active for many months. Similarly, Blakey et al. (1995) demonstrated that *Rhodococcus* cell pellets stored frozen at −20°C retained about 80% of their nitrile activity for over 20 months. The biomass in the vial could be defrosted in a 30°C oil bath while retaining activity.

Of the approximately 40 morphologically distinct environmental isolates, two (*Rhodococcus* ATCC BAA-870 and *Rhodococcus*

Fig. 1 Substrate structures



ATCC BAA-869) grew best on 3-hydroxy-3-phenylpropionitrile and were selected for further investigation. The carbon component of the selected nitrile substrates is not metabolised to any significant degree, as an accumulation of the amide and acid products in the defined medium is observed.

Biocatalytic hydrolysis of nitrile compounds

Biocatalysts

Organisms: Apart from the two isolates above, a *Rhodococcus globerulus* DSM 44519 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, provided by M. Wegman, TU Delft) and Novo SP361 Novozymes (Denmark), an immobilised *Rhodococcus* strain, were used as sources of nitrile hydratase activity.

Enzymes: A cell-free nitrilase preparation from *Pseudomonas fluorescens* DSM 7155 (a gift of Prof. Lutz Fischer of the Institute of Food Technology, University of Stuttgart-Hohenheim), a nitrilase from *Arabidopsis thaliana* (Jülich Fine Chemicals, Germany), and four other sources (BioCatalytics., USA) were included in the study.

Standard nitrile hydrolysis conditions

Layh et al. (1998) showed that the *P. fluorescens* DSM 7155 nitrilase has an optimum pH of 7–9, and is relatively stable to temperatures up to 55°C for short periods (30 min). Hence, the following general reaction conditions were chosen: A 10-μmol nitrile substrate dissolved in 100 μl methanol was added to 50 mM phosphate buffer, pH 7.2–7.4 (adjusted with HCl/NaOH) and a suitable quantity of biocatalyst. The final reaction volume of 2 ml was incubated at 30°C for 2 h with agitation (using a magnetic stirrer). A lower reaction temperature (<55°C) was used, as some α-substituted nitriles are thermolabile.

Methanol was selected as a substrate solvent as it is less denaturing than higher alcohols, and the nitrile hydratase of *R. equi* A4 was tolerant to 20% methanol (Prepechalová et al. 2001). Also, unlike DMSO, it did not obscure the (*R*)-phenylglycinamide peak when analysed on the Crownpak CR(+) column.

Samples of 1 ml reaction mixture were pipetted into Eppendorf snaplock vials (1.5 ml), and centrifuged (3,000 rpm for 5 min) using a microfuge to remove biomass. Aliquots of 200 μl reaction mixture supernatant were transferred to analytical vials containing 800 μl of the acidic HPLC eluent to halt the reaction.

Where required for chiral analysis, esterification of mandelic acid was achieved by reaction with BF_3-methanol in excess methanol, extraction into acetone, phase drying over $MgSO_4$, air drying, and re-suspension in suitable eluent.

Comparative evaluation of the substrate profile for nitrile biocatalysts

A range of nitrile compounds was evaluated as substrates for a set of nitrile-converting biocatalysts, including both whole-cell and cell-free systems.

Biocatalytic synthesis of carboxylic acids from the homologous aldehyde

Synthesis was carried out under standard reaction conditions using equimolar quantities of NaCN and aldehyde reactants in the presence of biocatalyst.

2-Hydroxy-3-phenylpropionamide could be biocatalytically synthesised from phenylacetaldehyde and HCN in the presence of a nitrilehydratase-active organism and the amidase inhibitor diethyl-phosphoramidate (DEPA, Aldrich) (Bauer et al. 1998). To determine the structure of the product the reaction was scaled up, centrifuged, acidified, extracted with 7× diethyl ether, washed over saturated brine, decanted, and phase dried over anhydrous Na_2SO_4. The volume was reduced by distillation at 50°C, and extracted back into water (3 volumes), and then into diethyl ether, dried by distillation, and examined by ^{13}C NMR. Although some DEPA was co-extracted, the product could be identified as 2-hydroxy-3-phenyl-propionamide by peak subtraction.

Protein determination

The protein concentration of cell-free extracts was determined by desalting on a P-10 desalting column (Pharmacia-Amersham), followed by protein determination using the method of Bradford (1976) (BioRad USA), with spectrophotometric quantification against bovine albumin fraction V standards.

Table 1 Conversion of arylaliphatic nitriles by biocatalysts. Figures in *parentheses* are the actual percentage conversion of phenylacetonitrile. *ND* Not determined

Biocatalyst	Compound										
	1	2	3	4	5	6	7	8	9	10	11
Pseudomonas fluorescens nitrilase	100% (97)	21%	89%	74%	1%	1%	0%	0%	11%	0%	0%
BioCatalytics nitrilase 1001	100% (16)	0%	0%	0%	0%	319%	0%	639%	12%	23%	0%
BioCatalytics nitrilase 1004	100% (87)	0%	0%	1%	0%	18%	0%	11%	3%	22%	2%
BioCatalytics nitrilase 1005	100% (95)	0%	0%	0%	0%	64%	0%	128%	62%	128%	128%
BioCatalytics nitrilase 1006	100% (100)	0%	14%	41%	0%	0%	0%	7%	3%	0%	0%
Arabidopsis thaliana nitrilase	100% (1)	0%	0%	0%	0%	4011%	0%	332%	278%	0%	0%
Rhodococcus ATCC BAA-870 nitrile hydratase	100% (14)	8%	5%	4%	10%	351%	167%	305%	118%	49%	37%
Rhodococcus ATCC BAA-869 nitrile hydratase	100% (22)	0%	0%	3%	0%	ND	9%	10%	0%	29%	11%
Rhodococcus DSM 44519 nitrile hydratase	100% (100)	46%	12%	9%	2%	ND	76%	83%	29%	100%	43%
Rhodococcus Novo SP361 nitrile hydratase	100% (100)	15%	4%	4%	10%	ND	30%	7%	9%	27%	13%

Results

Biocatalyst substrate profile

Each biocatalyst demonstrated a characteristic substrate-conversion profile on a wide range of substrates. As the purity of the enzymes and the specific enzyme content of the resting cells are unknown, the convention of reporting the data relative to a common activity [in this case phenylacetonitrile (**1**), Fig. 1)] has been used to define the substrate profile for each biocatalytic system (Table 1).

All the biocatalysts were active against the unsubstituted phenylacetonitrile (Fig. 1, **1**). The α-substituted arylaliphatic nitriles [2-phenylpropionitrile (**2**), 2-phenylglycinonitrile (**3**), mandelonitrile (**4**), and 2-phenylbutyronitrile (**5**), (Fig. 1)] were hydrolysed to various degrees by the nitrile hydratase systems of the *Rhodococcus* biocatalysts but were generally unsuitable for the nitrilases, and only the *P. fluorescens* nitrilase and Nitrilase-1006 (BioCatalytics) exhibited activity for this type of substrate. Even the enzymes that accepted α-substituents were slow to convert the bulkier 2-phenylbutyronitrile (**5**).

The yield of 2-phenylglycine from 2-phenylglycinonitrile (**3**) was reduced by a few percent due to the limited spontaneous decomposition of the substrate under the reaction conditions to yield benzaldehyde and (by a spontaneous reaction of free cyanide with benzaldehyde) mandelonitrile (**4**). As the enzyme is also active against the latter compound, some of this was subsequently converted to mandelic acid.

The unsubstituted compounds with longer aliphatic groups (3-phenylpropionitrile, **6**) and the structural homolog (*N*-phenylglycinonitrile, **8**) were generally accessible to all of the biocatalysts and were preferred substrates for nitrilases 1001, 1005, and particularly that of *A. thaliana*. However, for the β-substituted propionitrile compound (±)-3-hydroxy-3-phenylpropionitrile (**7**), only the nitrile hydratases were capable of hydrolysis, with the nitrilases showing no activity. The highest relative activity on this substrate was demonstrated by *Rhodococcus* ATCC BAA-870, which is to be expected as it was the substrate on which the organism was isolated.

The aromatic nitriles [phthalonitrile (**9**), *p*-toluonitrile (**10**), and 3,4-dimethoxybenzonitrile (**11**)] were all hydrolysed by the nitrile hydratase systems, which possessed a very broad substrate range. Surprisingly, in spite of a preference for longer aliphatic groups in arylaliphatic nitriles, the nitrilase preparations generally had activity against some of the aromatic nitriles, particularly nitrilase 1005 (BioCatalytics; Table 1).

Nitrilases 1001 and 1005 preferred propionic aliphatic groups without α- or β-substitution. Nitrilase 1004 was relatively specific for unsubstituted phenylacetonitrile, while 1006 and *P. fluorescens* nitrilases had activity against α-substituted phenylacetonitriles. *Arabidopsis thaliana* nitrilase (Nit 1) mediated the hydrolysis of 3-phenylpropionitrile or compounds with a similar chain length without (**6, 8**), but not with (**7**) α- or β-substitution (Table 1).

The *Rhodococcus* cells were the only biocatalysts capable of converting the β-hydroxynitrile compounds to the corresponding β-hydroxy carboxylic acids (Table 1). Evidently, the capacity for this reaction is common among the nitrile hydratases, but not the nitrilases.

The nitrilases only generated the corresponding carboxylic acid, and no significant quantities of the amide were produced. The *Rhodococcus* biocatalysts generated both the amide and the acid, and were thus revealed to be predominantly nitrile hydratase/amidase systems (Table 2). However, the ratio of acid to amide produced was variable, being dependent on the microbial species, the substrate structure, the incubation time, and the culture batch (the amount of amidase apparently varying greatly with culture age). Based on the frequently observed accumulation of amides, it can be concluded that either the expression or the activity of amidases for the substrates investigated in this study seems to be considerably lower than that of the nitrile hydratase, as observed previously in these laboratories (Wegman et al. 2000).

Other compounds of interest were investigated using a smaller range of biocatalysts. The *P. fluorescens* DSM 7155 nitrilase was able to completely and rapidly convert 4-nitrophenylacetonitrile (**12**) and bromophenylacetonitrile (**17**), but was capable of only extremely slow conversion of 1-*H*-benzoimidazol-2-yl-acetonitrile (**13**) at 20% hydrolysis in 15 h. 3-(3,5-Dimethyl-pyrazol-1-yl)-propionitrile (**16**) was not converted, nor was this nitrilase able to convert significant quantities of hydroxy-(4-methoxy-3-methyl-phenyl)acetonitrile (**14**), or 2-acetyl-phenylacetonitrile (**15**), possibly due to bulky groups in certain positions on the aromatic ring.

Rhodococcus ATCC BAA-870 was able to convert 3-(3,5-dimethylpyrazol-1-yl)-propionitrile (**16**) at a rate of 0.125 μmol (mg dry mass)$^{-1}$ min^{-1}, confirming the

Table 2 Degree of complete hydrolysis of nitriles to the carboxylic acid. - No conversion,*ND* not determined

Biocatalyst	Substrate								
	1	2	3	4	6	7	8	9	11
P. fluorescens nitrilase	100%	100%	94%	100%	100%	-	-	-	-
Rhodococcus ATCC BAA-870 nitrile hydratase	3%	0%	0%	21%	ND	3%	0%	21%	0%
Rhodococcus ATCC BAA-869 nitrile hydratase	2%	-	-	47%	0%	28%	0%	12%	0%
Rhodococcus DSM 44519 nitrile hydratase	12%	9%	0%	8%	ND	16%	52%	28%	3%
Rhodococcus Novo SP361 nitrile hydratase	24%	35%	0%	0%	ND	32%	77%	83%	44%

preference for a propionitrile group. For comparison, 3-hydroxy-3-phenylpropionitrile (**7**) was converted at a rate of 0.21 μmol mg^{-1} min^{-1}, and *N*-phenylglycinonitrile (**8**) was converted at 0.29 μmol mg^{-1} min^{-1} (Table 3).

Stereoselectivity

In the current study, a 26% ee of (*R*)-mandelic acid at 64% conversion from mandelonitrile was obtained using the *P. fluorescens* DSM 7155 nitilase. Similarly, Layh et al. (1998) used the same nitrilase for kinetic resolution of 2-methoxymandelonitrile. Although good enantioselectivity at 92% ee (*R*-acid) was obtained at 50% conversion, this was reduced to 27% ee (*R*) at 85% conversion. This ee indicates that the reactant racemises spontaneously during the reaction (otherwise the achievable ee would be <18% at 85% conversion).

The nitrile hydratase of *Rhodococcus* ATCC BAA-870 had an unusually high activity for 3-hydroxy-3-phenyl-propionitrile relative to phenylacetonitrile, but failed to demonstrate any enantioselectivity in this reaction, while *R. globerulus* DSM 44519 did show a slight enantioselectivity (14% ee). Poor enantioselectivity for the hydrolysis of (*R*,*S*)-2-phenylpropionitrile and (*R*,*S*)-2-phenylbutyronitrile by the *Rhodococcus* catalyst Novo SP 361 (7% and 0% ee, respectively) was observed.

Chemoselectivity

One of the purported advantages of nitrile biocatalysis over the classical hydrolysis under extreme pH is the ability to selectively facilitate this hydrolysis without affecting other acid- or alkali-labile functional groups present on the same molecule. To demonstrate this capacity, a nitrile compound containing ester bonds (**18**) was selectively hydrolysed by the *P. fluorescens* nitrilase to yield the mono-carboxylic acid ethyl ester. No other hydrolysis products were observed by HPLC, although the product is a hemi-malonic acid and hence prone to decomposition at low pH. This unexpectedly implies that, even though activity against 2-phenylbutyronitrile was low, the active site of the *P. fluorescens* nitrilase can accommodate relatively bulky α-substituents. Further research using similar substrates is needed to confirm this conclusion using purified nitrilase.

Although no hydrolysis of the model ester (2-hydroxy-3-phenylpropionic acid ethyl ester) was observed in a control experiment, the benzoate ester of mandelonitrile (**19**) was hydrolysed, presumably by an esterase activity, to mandelonitrile and benzoic acid (rather than the expected half ester of phenylmalonic acid). The mandelonitrile was subsequently hydrolysed by the *P. fluorescens* nitrilase to mandelic acid. It is known that whole cells or crude cell-free preparations may contain esterase activity (Faber 1992), while Sugai et al. (1997) commented on the presence of esterases in nitrile-hydrolysing *Rhodococcus* strains.

Regioselectivity

The regioselective nature of nitrile-converting enzymes was investigated using dinitrile compounds as substrates (**20**, **21**, **22**, **23**). 4-Cyanomethylphenylacetonitrile (**20**) was converted by the *P. fluorescens* nitrilase to the mono-acid mono-nitrile (while *Rhodococcus* ATCC BAA-870 generated both the amide and the acid). 2-Cyanomethyl-phenylacetonitrile (**21**) and phthalonitrile (**22**) were converted into the mono-acid mono-nitrile compound, while 4-nitrophthalonitrile (**23**) was converted into both of the possible mono-acid mono-nitriles (**24**, **25**), and hence the reaction was therefore not strictly regioselective.

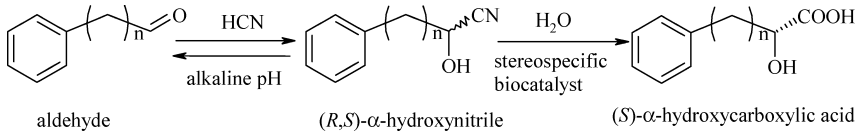
Biocatalytic synthesis of α-substituted carboxylic acids from the homologous aldehyde

Synthesis of α-hydroxycarboxylic acids and amides from the structurally related aldehydes and ketones, via cyano-hydrin intermediates, was attempted. Under alkaline conditions, (e.g. pH 8.5) cyanide ion adds to aldehydes to yield the racemic α-hydroxynitrile (Wieser and

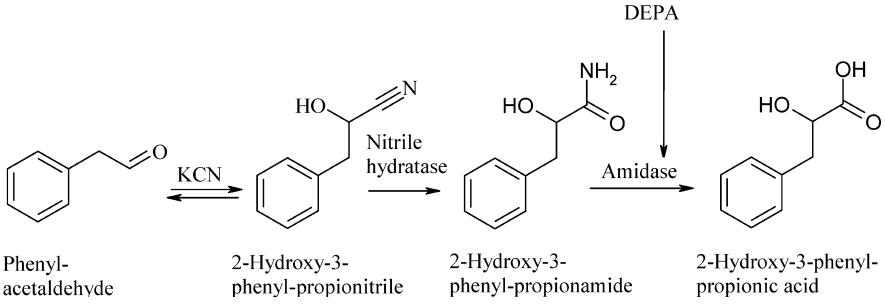
Table 3 Initial kinetic rates of nitrile hydrolysis (μmol mg^{-1} min^{-1})

Biocatalyst	Substrate										
	1	2	3	4	5	6	7	8	9	10	11
P. fluorescens nitrilase	0.08	0.57	5.90	1.12	0.04	0.05	0	0	0.01	0	0
BioCatalytics nitrilase 1001	0.01	0	0	0.00	0	0.43	0	0.51	0.002	0.003	0
BioCatalytics nitrilase 1004	0.11	0	0	0.10	0	0.02	0	0.01	0.01	0.02	0.001
BioCatalytics nitrilase 1005	0.10	0	0	0.00	0	0.85	0	0.95	0.01	>2.0	0.33
BioCatalytics nitrilase 1006	0.25	0	0.01	0.28	0	0	0	0.01	0.01	0	0
Arabidopsis thaliana nitrilase	0.001	0	0	0.00	0	0.01	0	0.01	0.01	0	0
Rhodococcus ATCC BAA-870 nitrile hydratase	0.19	0.01	0.01	0.02	0.003	0.01	0.21	0.29	0.02	0.005	0.004
Rhodococcus ATCC BAA-869 nitrile hydratase	0.30	0	0	0.01	0	ND	0.02	0.02	0	0.01	0.002
Rhodococcus DSM 44519 nitrile hydratase	0.08	0.03	0.01	0.00	0.002	ND	0.06	0.06	0.03	0.08	0.03
Rhodococcus Novo SP361 nitrile hydratase	0.04	0.01	0.0016	0.00	0.004	ND	0.01	0.00	0.01	0.03	0.02

Scheme 4 Biocatalytic synthesis of α-hydroxy carboxylic acids



Scheme 5 The multi-step transformation of phenylacetaldehyde to 2-hydroxy-3-phenylpropionic acid can be halted at the amide intermediate by addition of diethyl-phosphoramidate (DEPA)



Nagasawa, 2000). This in turn may be stereoselectively converted to the homologous acid by a nitrilase (Scheme 4). Although cyanide is a potential inhibitor, it is known that the *P. fluorescens* DSM 7155 nitrilase is only slightly inhibited by KCN at 10 mM (Layh et al. 1998).

In the present study, addition of mandelonitrile to the reaction mixture resulted in spontaneous elimination to yield the cyanide and benzaldehyde in dynamic equilibrium with the mandelonitrile itself. With addition of biocatalysts, almost complete conversion to mandelic acid was possible with quantitative yields, demonstrating the spontaneous elimination reaction to be reversible under these conditions. The biocatalytic formation of mandelic acid from benzaldehyde and cyanide in the presence of a nitrilase has been previously observed by Yamamoto et al. (1991), and 2-methoxymandelic acid from 2-methoxybenzaldehyde and cyanide by Layh et al. (1998). In the present study, a range of aldehydes and ketones was investigated as substrates for this two-step reaction. The results (Table 4) demonstrate that the two-step synthesis from aldehyde to the homologous α-hydroxycarboxylic acid is possible when the biocatalyst has a preference for the α-hydroxynitrile (as was also recently observed by DeSantis et al. 2002).

Similar to the experiments with nitriles, the HCN adducts of aldehydes with bulky aromatic substituents were not hydrolysed to the acid by the *P. fluorescens* nitrilase, for example vanillin (**26**), 4-methoxybenzaldehyde (**27**), and 4-methylsulphoxybenzaldehyde (**28**) (Table 4). Aminophenylacetaldehyde (**29**), phenylacetaldehyde (**30**), and 3-phenylpropionaldehyde (**31**) were also not converted by the *P. fluorescens* nitrilase. Based on its broad substrate range, it was predicted that *Rhodococcus* ATCC BAA-870 would be able to yield 2-hydroxy-3-phenylpropionic acid by in situ synthesis from phenylacetaldehyde (**30**) in the presence of cyanide.

When 4-formylbenzonitrile was incubated in the presence of cyanide, yielding 4-(cyano-mandelonitrile), one of two conversions occurred, depending on the catalyst added. Nitrilase 1005 could not hydrolyse the aliphatic nitrile (as demonstrated in a similar experiment in which 4-methyl benzaldehyde was the added aldehyde) but could hydrolyse the aromatic nitrile, and hence yielded 4-formylbenzoic acid and 4-cyanomandelic acid. Alternatively, the *P. fluorescens* nitrilase had poor activity towards aromatic nitriles (e.g. *p*-toluonitrile, Table 1) and yielded a single product peak on HPLC, corresponding to 4-cyanomandelic acid.

Table 4 Biocatalytic synthesis of α-hydroxy carboxylic acid from α-hydroxy nitrile by *P. fluorescens* nitrilase

Aldehyde compound incubated with cyanide and biocatalyst	Conversion to homologous α-hydroxy acid (%)
benzaldehyde	95
4-methylbenzaldehyde	51
4-hydroxybenzaldehyde	55
2-nitrobenzaldehyde	20
2-fluorobenzaldehyde	100
3-chlorobenzaldehyde	100
4-chlorobenzaldehyde	100
4-nitrobenzaldehyde	100
2-chlorobenzaldehyde	90
Vanillin	0
4-methoxy-benzaldehyde (p-anisaldehyde)	0
4-cyanobenzaldehyde	100
4-methylsulphoxybenzaldehyde	0
Trimethoxybenzaldehyde	0

The HCN adducts of ketones did not afford 2-methyl-2-hydroxycarboxylic acids, perhaps due to the steric hindrance of the tertiary substituted α-carbon. Acetophenone (**32**), and phenylacetone (**33**) were not converted to the acid or amide by the *P. fluorescens* nitrilase, nitrilase 1006 or by Novo SP361. The *P. fluorescens* nitrilase apparently was incapable of competing kinetically with the observed spontaneous degradation of the HCN adduct to acetophenone.

2-Hydroxy-3-phenylpropionic acid could be synthesised from phenylacetaldehyde (30) and HCN in situ by *Rhodococcus* ATCC BAA-870 (Scheme 5). This reaction

could be halted at the amide intermediate by the addition of diethyl-phosphoramidate (DEPA, Aldrich), a compound that is known to be an amidase inhibitor (Bauer et al. 1998).

Strecker synthesis of a number of phenylglycine homologs with aromatic substituents was carried out. However, with nitro-substituted benzaldehydes an exothermic polymerisation reaction took place and dark-conjugated by-products were formed even when the reaction was cooled. The α-amino nitrile compounds hydrolysed into the acid by *P. fluorescens* nitrilase in the standard reaction were those derived from benzaldehyde, 4-hydroxybenzaldehyde, and 3- and 4-chlorobenzaldehyde, yielding phenylglycine and its aromatic substituted analogs.

Alternative nucleophiles

Nitrilases 1001, 1004, 1005, 1006 and that from *P. fluorescens* were incubated in the presence of nitriles and the nucleophiles hydroxylamine or hydrazine (according to the method of Hirrlinger et al. 1997) but failed to utilise these alternative nucleophiles.

Discussion

That the great number of nitrile-hydrolysing bacterial species isolated from the environment were from the genus *Rhodococcus* was no surprise, as similar profiles have been noted previously (Bunch 1998). The *Rhodococcus* sp. all demonstrated nitrile hydratase activity, with broad substrate specificity. They (particularly those isolated in these laboratories) were capable of transforming β-substituted arylpropionitriles, which is in stark contrast to the nitrilases. However, the *Rhodococcus* sp. were poorly enantioselective for this substrate, limiting their immediate synthetic application.

Layh et al. (1998) defined nitrilases as fitting into three sub-classes, depending on their substrate affinity:

1. Aliphatic nitrilases (exclusive), e.g. *Rhodococcus rhodochrous* K22 nitrilase.
2. Aromatic nitrilases that hydrolyse aromatic and heterocyclic nitriles, e.g. those enzymes from *Arthrobacter* sp.
3. Arylacetonitrilases, which predominantly catalyse the hydrolysis of arylacetonitriles, e.g. those activities from *Alcaligenes faecalis*, *Arabidopsis thaliana*, and *Pseudomonas fluorescens* DSM 7155 (Yamamoto et al. 1991; Layh et al. 1998; Effenberger and Osswald 2001a).

The biocatalysts in this study appear to be functional arylacetonitrilases. The *P. fluorescens* DSM 7155 nitrilase preferred acetonitriles, and accepted various α-substitutions and aromatic substitutions. Layh et al. (1998) found that the arylacetonitrilase nitrilase from *P. fluorescens* DSM 7155 converted (relative to phenylacetonitrile) 100% phenylacetonitrile, 2% 2-phenylpropionitrile, and 0.1% 2-phenylbutyronitrile, the decreasing conversion ascribed to the increasing bulk of the α-substituent. The aromatic nitriles benzonitrile and 2-cyanotoluene were not hydrolysed under these conditions. In the present study a similar order of activity was observed: 100% phenylacetonitrile, 21% 2-phenylpropionitrile (α-methylphenylacetonitrile), and 1% 2-phenylbutyronitrile.

The lack of activity by many of the nitrilases investigated here against α-substituted phenylacetonitriles seems common in this class of enzyme, as the *Bacillus* sp nitrilase investigated by Almatawah et al. (1999) had no activity against mandelonitrile. Similarly, Osswald et al. (2002) noted that hydrolysis of the nitrile of 2-phenylpropionitrile by the *Arabidopsis thaliana* nitrilase was completely inhibited by the bulky α-substituent. The exception was 2-fluoro compounds, presumably due to the small size of this substituent. Aromatic nitriles, such as benzonitrile, were hydrolysed at less than 1% the rate of 3-phenylpropionitrile, and phenylacetonitrile at less than 5% the rate of 3-phenylpropionitrile (Osswald et al. 2002).

Although many nitrile hydratases have been reported to prefer aliphatic nitriles, those from organisms such as *R. rhodochrous* J1 have high activity towards aromatic nitriles (Wieser and Nagasawa 2000). The immobilised whole-cell catalyst Novo SP361 nitrile hydratase demonstrated regioselectivity for converting aromatic nitriles to the corresponding acid, but was also capable of hydrolysing aliphatic nitriles (Cohen et al. 1990). This catalytic capacity of *Rhodococcus* Novo SP361 for a broad substrate range was also observed in the current study, including accepting α- or β-substitution on the aliphatic chain of arylpropionitriles. Similarly, the *Rhodococcus* nitrile-degrading biocatalyst Novo SP409 was used by Klempier et al. (1991) for the conversion of aliphatic β-hydroxynitriles without elimination or epimerisation. The broad substrate range of the *Rhodococcus* sp. may be partially due to the presence of more than one nitrile hydratase (Wieser et al. 1998).

Consistent with the present study, Novo SP 361 did not demonstrate enantioselectivity for the hydrolysis of (*R*,*S*)-2-phenylpropionitrile and (*R*,*S*)-2-phenylbutyronitrile (Beard et al. 1993; Bauer et al. 1998). Prepechalová et al. (2001) found no stereoselectivity of *R. equi* A4 for 2-phenylbutyonitrile. Other strains may perform better with this substrate; Blakey et al. (1995) determined that the nitrile hydratase from *Rhodococcus* AJ270 was stereospecific for hydrolysis of (*R*)-2-phenylbutyronitrile to (*R*)-2-phenylbutyramide. Fallon et al. (1997) catalysed the hydrolysis of 2-(4-chlorophenyl)-3-methylbutyronitrile with a nitrile hydratase and obtained 90% enantiomeric excess (ee) of the *S*-amide.

In agreement with our results, hydrolysis of 4-methylbenzonitrile (*p*-toluonitrile) by *Rhodococcus sp.* has been observed by Dadd et al. (2001b).

Generally, the nitrilases and nitrile hydratases in microbial isolates have shown relatively poor enantioselectivity, while the amidases have shown strong (*S*)-selectivity for amides, with ee >99% (Layh et al. 1997;

Martínková et al. 1996)). However, it has been suggested that the aromatic moiety of nitriles of profens facilitates enantiospecific recognition since enantioselective nitrile-converting biocatalysts have been more common for these compounds (Wieser and Nagasawa 2000). That the stereoselective hydrolysis of ring-substituted α-methyl-phenylacetonitriles by immobilised *Rhodococcus* sp. was sensitive to the aromatic ring substituent (Beard et al. 1993; Sugai et al. 1997; Prepechalová et al. 2001; Martínková and Křen 2002; Martínková et al. 1996) reinforces this perception.

(*R*)-α-Phenylglycine is of commercial interest as a raw material for cephalosporins (Wieser and Nagasawa, 2000). Wegman et al. (2001) showed that a *Rhodococcus* strain (DSM 44519) converted 100% of D,L-phenylglycine to the amide without stereoselectivity, but only the (*S*)-enantiomer was converted to the acid by the highly stereospecific amidase (>99% ee). The activity of the nitrile hydratase was, however, synthetically beneficial as it permitted stereoretentive hydrolysis to the amide without the racemisation and decomposition of the substrate that would occur under chemical hydrolysis conditions.

In this investigation it appears that in each case only one of the nitrile groups was hydrolysed in aryl and aliphatic dinitriles. Other authors have also observed that 1,3- and 1,4-dicyanobenzenes were selectively hydrolysed by *R. rhodochrous* to give the corresponding mono-acids (Faber 1992). Cohen et al. (1990) obtained mono-nitrile mono-acids of 1,3 and 1,4 benzodinitriles using Novo SP361, but phthalonitrile was converted to the diacid. Application of *Rhodococcus* ATCC BAA-870 to 4-cyanomethylphenylacetonitrile (**20**) yielded results similar to those of Effenberger and Graef (1998), who found that *Rhodococcus erythropolis* C3II first generated the mono-nitrile mono-amide by partial hydrolysis of 4-cyanomethylphenylacetonitrile, followed by conversion to the mononitrile monoacid by a slower acting amidase.

Interestingly, with the appropriately stereospecific biocatalyst, racemic α-substituted carboxylic acids and amide compounds can be generated as single enantiomers from the nitrile intermediate by means of dynamic kinetic resolution. Such enzymes are available, as 2-chloro-, 4-chloro-, 4-bromo-, 2-fluoro-, 4-methyl-, 4-methoxy-, 4-methylthio-, and 4-nitro-(*R*)-mandelic acid derivatives have been enantiospecifically produced by nitrilase biocatalysts from the substituted mandelonitrile (Wieser and Nagasawa 2000). Using whole-cell *Alcaligenes faecalis* ATCC 8750 biocatalysts, Yamamoto et al. (1991) achieved 100% ee and 91% of the theoretical yield of (*R*)-mandelic acid. The capacity to generate α-hydroxy acids (many of which are commercially valuable) in high yields without co-production of the unwanted enantiomer would have commercial benefits (Yamamoto et al. 1991: Wieser and Nagasawa 2000).

The generation of α-hydroxy amides and acids from the constituent aldehydes and cyanide in situ would allow for many useful syntheses at good kinetic rates. The inability to yield α-di-substituted compounds from ketones warrants further research, including the selection of organisms for this reaction.

The Strecker method may be used in a one-pot reaction wherein the initial chemical synthesis step, yielding an α-amino nitrile, is followed by dilution and hydrolysis with nitrilase to the α-amino carboxylic acid (Weiner and Chaplin 2000). Even more advantageous would be the possibility of inclusion of the enzyme in the Strecker reaction. However, in the present study this was problematical as the activity was lost at the high salt concentrations, and aqueous dilution resulted in increased levels of the α-hydroxy by-product. The poor performance of the nitrilases in in-situ Strecker/hydrolysis reactions may be due to inhibition or enzyme denaturation. The latter effect may be at least partially overcome by immobilisation of the enzyme. Such immobilised nitrilases are being prepared in our Delft laboratories.

The apparent inability of nitrilases to utilise alternative nucleophiles supports the findings of others (Kobayashi et al. 1999; Dadd et al. 2001a; Hirrlinger et al. 1997) that this capacity resides primarily in amidases rather than nitrilases or nitrile hydratases.

The aim of the current study was to determine the substrate profiles of a range of nitrile-hydrolysing biocatalysts. Knowledge of such catalysts should facilitate more directed investigation into their application for specific syntheses. It is evident from this report that specific catalysts, or combinations thereof, could be used to selectively generate acids and amides from compounds with more than one nitrile group, depending on aliphatic chain lengths and substitution.

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