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Characterization and partial purification of an enantioselective arylacetonitrilase from *Pseudomonas fluorescens* DSM 7155

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Abstract

Pseudomonas fluorescens DSM 7155 after growth on phenylacetonitrile as sole nitrogen source contained an inducible nitrilase which consists of two different functional subunits (40 and 38 kDa). The nitrilase catalysed the exclusive hydrolysis of arylacetonitrile substrates into the equivalent carboxylic acids plus ammonia as major products. The corresponding amides were formed at low levels (< 5%) during nitrile hydrolysis but were not substrates for the purified enzyme. The native enzyme, which had a pH optimum of 9 and a temperature optimum of 55°C, was activated (140–160%) by the thiol protectant 2-mercaptoethanol (50–100 mM). The purified nitrilase catalysed the hydrolysis of the two enantiomers of racemic 2-(methoxy)-mandelonitrile to the corresponding acid at significantly different rates: at 50% overall conversion the predominant product was the (*R*)-acid (enantiomeric excess = 92%) whereas at 85% overall conversion the ee% of the (*R*)-acid had decreased to 27%. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Nitrilase; Arylacetonitrilase; Chiral carboxylic acid; *Pseudomonas fluorescens*; Enzyme purification

1. Introduction

Nitrilases are the subject of much attention because of their potential as biocatalysts for the production of higher-value acids [1]. Resting cells from *Rhodococcus rhodochrous* J1 have been used successfully for the production of both *p*-aminobenzoic acid and nicotinic acid from the corresponding nitriles [2,3]. Nitrilases have also been applied successfully to catalyse the regiospecific hydrolysis of dinitrile compounds into corresponding cyano-carboxylic acids [4,5]. With the exception of the nitrilase from *Rh. rhodochrous* PA-34 [6] and an *Arthrobacter* sp. [7] which consist of a single polypeptide acting as a monomer, all other nitrilases characterised to data are homopolymers often containing substantial numbers (6–16) of the relevant component subunit [8].

In this paper, we report the purification and properties of a novel hetero-oligomeric enantioselective arylacetonitrilase from *Pseudomonas fluorescens* DSM 7155.

2. Materials and methods

2.1. Materials

Phenyl-Sepharose FF, Mono Q, Superose 12 and the reference proteins used to determine

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molecular weight were purchased from Pharmacia (Sweden). All other chemicals used were from commercial sources except phenylacetamide which was prepared by T. Stock (Chemistry, Exeter) and 2-(methoxy)-mandelonitrile plus 2-(methoxy)-mandelic acid which were provided by J. Parratt (Chiroscience, Cambridge).

2.2. Microorganisms and culture conditions

P. fluorescens DSM 7155 which was previously isolated from soil (Synonym: *P. fluorescens* EBC191; [9]) was used as a source of the enzyme for the purification.

The nitrogen-free culture medium (pH 7.4) contained 14 g $Na_2HPO_4 \cdot 12\ H_2O$, 2 g KH_2PO_4, 0.05 g $CaCl_2 \cdot 2\ H_2O$, 0.05 g Fe^{3+}-citrate, 0.02 g $MgSO_4 \cdot 7\ H_2O$ and 1 ml of a trace element solution described by [10] without iron salts and EDTA. A culture that had first been grown in 100 ml medium with succinate (10 mM) as principal source of carbon and phenylacetonitrile (1.5 mM) as both the sole source of nitrogen and an inducer for the nitrilase was inoculated into 800 ml of the same medium in a 2-l Erlenmeyer flask and cultured at 30°C with reciprocal shaking (250 rpm). After 24 h, when the cells were in the mid-exponential phase of growth, they were harvested by centrifugation ($6500 \times g$, 4°C, 30 min).

2.3. Analysis

Protein was determined by the Bradford method [11], using bovine serum albumin as a standard. The decrease of nitrile substrates and the formation of corresponding product(s) were analysed by HPLC using a Spherisorb S5 ODS2 column (4.6 × 200 mm) attached to a Shimadzu LC-10AS pump and a Pye-Unicam LC3 detector (210 nm wavelength). The eluting solvent usually contained 30%–60% (v/v) methanol plus 0.2% (v/v) phosphoric acid, depending on the nitriles being tested. Chiral analysis of 2-(methoxy)-mandelic acid was carried out at Chiroscience (Cambridge, UK) by chemical formation of the methyl ester followed by separation on a Chirasil Dex-CB column (carrier gas: Helium at 20 psi and 130°C). Under these conditions the enantiomers had retention times of 22.0 and 23.4 min, respectively. The predominant enantiomer was confirmed by comparison with a known authentic standard.

2.4. Enzyme assay

Nitrilase activity was routinely assayed with phenylacetonitrile as the substrate. The standard reaction was performed at 30°C in a reaction mixture (100 μl) containing 5 μmol Tris/HCl pH 7.4, 10 μmol 2-mercaptoethanol, 1 μmol nitrile and an amount of enzyme equivalent to 0.01 U. To start the biotransformation, the nitrile was added in form of a stock solution (200 mM in methanol). Samples of the reaction mixture (100 μl) removed at various times were stopped by the addition of 10 μl 1 M HCl. After spinning down to remove precipitated protein (4 min, 13 000 rpm), the resultant supernatant was in each case analysed by HPLC. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of carboxylic acid per minute in the reaction mixture.

2.5. Purification of nitrilase

All purification steps were performed at 0–4°C. The buffer solution used throughout was 50 mM Tris/HCl buffer pH 7.4 containing 100 mM 2-mercaptoethanol.

2.5.1. Step 1. Preparation of a cell-free extract

Cells from 10 l spent culture broth were suspended in 65 ml Tris/HCl buffer (50 mM, pH 7.4) containing 100 mM 2-mercaptoethanol and then disrupted by sonification as already described [12]. The cell debris was removed by centrifugation at $60\,000 \times g$ for 30 min.

2.5.2. Step 2. Phenyl-Sepharose FF column

To the cell-free extract ammonium sulfate was added to a final concentration of 100 mM.

After 2 h stirring at 4°C, the precipitated protein was filtered (Minisart 0.2 μm, Sartorius, Germany) and applied on a Phenyl Sepharose FF column (2 × 5.5 cm) equilibrated with buffer containing 100 mM ammonium sulfate. The column was washed once with one volume of the same buffer, and then the enzyme was eluted with buffer containing 20% (v/v) ethylene glycol. The nitrilase-active fractions were combined for further purification.

2.5.3. Step 3. Mono Q column

The active enzyme from step 2 was loaded onto a MonoQ column equilibrated with Tris/HCl buffer. The nitrilase was eluted with a linear gradient of NaCl (0–0.8 M) in the same buffer. The nitrilase-active fractions were combined and concentrated (Centricon 3, Amicon, USA).

2.5.4. Step 4. Superose 12 column

The concentrated nitrilase solution was applied to a Superose 12 column, equilibrated with Tris/HCl buffer containing 50 mM NaCl and then eluted with the same buffer.

2.6. Analysis of the purified nitrilase

The molecular mass of the protein was calculated by comparison with the relative mobilities of standard proteins on the Superose 12 column. SDS/PAGE was performed in homogeneous 12.5% (w/v) polyacrylamide gels (PhastGel, Pharmacia, Sweden) using a Pharmacia LKB PhastSystem.

2.7. Amino acid sequencing

After running a SDS-gel of the purified enzyme eluted from the Superose 12 column, the three resultant protein bands were each electroblotted onto a separate immobilisation membrane as previously described [13]. The sequencing of each band was then carried out by the Department of Biochemistry, University of Aberdeen, UK.

3. Results

Nitrilase from *P. fluorescens* DSM 7155 was only present after growth on defined media containing phenylacetonitrile as sole nitrogen source. The use of either ammonia as a replacement nitrogen source in the minimal medium or a complex medium such as nutrient broth caused a substantially higher cell yield (approximately 4-fold), but no nitrile hydrolysing activity could be detected in such cells. Interestingly, the presence of both ammonia plus phenylacetonitrile in minimal media also resulted in good cell yields but again with no detectable nitrilase activity suggesting some form of N-catabolite repression. Similarly, supplementation of growth media with ε-caprolactam (0.5% w/v), reported as a strong inducer for nitrilase in several *Rhodococcus* strains and *Alcaligenes faecalis* JM3 [14], resulted in no detectable induction of the enzyme.

3.1. Purification of nitrilase

The nitrilase from *P. fluorescens* DSM 7155 was purified 259-fold from the cell-free extract of cells grown on phenylacetonitrile-based minimal medium (Table 1). The most highly purified preparation appeared as a single symmetrical protein peak (130 kDa) on gel filtration from

Table 1
Purification of nitrilase from *P. fluorescens* DSM 7155

Step	Protein [mg]	Total activity [U]	Specific activity [U/mg]	Yield [%]	Purification [fold]
Crude extract	140	48	0.347	100	1
Phenyl sepharose FF	6	27	4.5	57	13
Mono Q	0.465	12.8	27.5	27	79
Superose 12	0.055	5	90	10	259

1 MSSNPELKYTKGVKVATVQA

2 M - - - - - VEYTNTFKVAAVQA

3 MDTT - - - - - - - - FKAAAVQA

4 MQTR - - - - - - K IVRAAAVQA

5 MQTR - - - - - - KLVRAAAVQA

6 - Q - - - - VHK-KQEKYSA?Q

7 - Q - - - - VHK-KQYKVA?QQ

1 Nitrilase from *Rhodococcus rhodochrous* K22 [16]
2 Nitrilase from *Rhodococcus rhodochrous* J1 [15]
3 Nitrilase from Klebsiella pneumoniae [31]
4 Nitrilase from *Alcaligenes faecalis* ATCC 8750 [24]
5 Nitrilase from *Alcaligenes faecalis* JM3 [30]
6 38kDa nitrilase subunit from *Pseudomonas fluorescens* DSM 7155
7 40kDa nitrilase subunit from *Pseudomonas fluorescens* DSM 7155

Spacing (-) has been introduced to allow for greater sequence homology between the different proteins

Fig. 1. N-terminal sequence data for the 40 kDa and 37 kDa subunits isolated from *P. fluorescens* DSM 7155 nitrilase and various other equivalent proteins [15,16,24,30,31]. Spacing (-) has been introduced to allow for greater sequence homology between the different proteins.

the Superose 12 column, but fractionated into three different bands on SDS-polyacrylamide gel electrophoresis. When relevant samples were examined by SDS-PAGE, it was apparent that these three bands were always enriched to the same relative extent during each step of the purification protocol. The molecular weight of the three peptide subunits (57, 40 and 38 kDa) corresponded well with that of the native protein (130 kDa).

The results obtained from the N-terminal amino acid sequencing were interesting. Firstly, the 57 kDa fragment shared almost total identity (97% homology) with the chaperonin CPN60 in the 30 residues of the N-terminus that were determined. The two lower molecular weight peptides (40 kDa and 38 kDa) showed considerable homology (62%) in the 13 residues that were determined, and are clearly related to each other. Both peptides showed significant sequence homology to the N-terminus of several other previously characterised nitrilases (Fig. 1), which appears to be a common feature of these enzymes sourced from a wide range of bacteria [14–17].

3.2. Effects of pH and temperature

The effects of different reaction conditions on the activity of the purified native nitrilase were studied (Fig. 2A). A broad pH curve with maximum activity at pH 9 was found. The

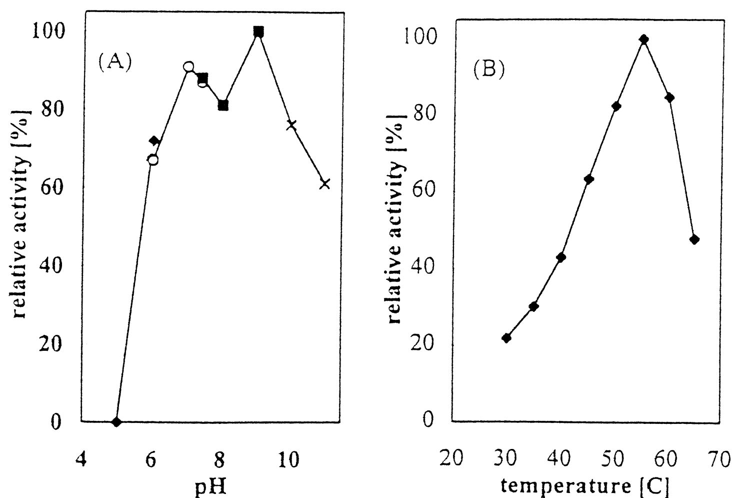

Fig. 2. Effects of pH (A) and temperature (B) on the activity of purified nitrilase. (A) Reactions were run for 20 min at 30°C in the following 0.1 M buffers: Succinic acid/NaOH (♦), Na/K phosphate (○), Tris/HCl (■), cyclohexylaminopropanesulfonic acid/NaOH (x). (B) The reactions were performed out for 30 min at various temperatures. Relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions.

optimum temperature for nitrile hydrolysing activity was around 55°C.

3.3. Substrate specificity

The ability of the purified native nitrilase to catalyze the hydrolysis of various nitriles to equivalent carboxylic acids was examined and the relative activities obtained are listed in Table 2. The enzyme preferentially hydrolysed phenylacetonitrile but no detectable activity was recorded with any of the tested aromatic, heterocyclic or aliphatic nitriles, indicating that this enzyme is an arylacetonitrilase. In support of this conclusion, it is known that washed-cell preparations of *P. fluorescens* DSM 7155 are also capable of hydrolysing mandelonitrile, *O*-acetylmandelonitrile, α-(methoxy)-phenylacetonitrile and α-(amino)-phenylacetonitrile (R. Bauer, personal communication). No activity was detected with a range of tested amides, including phenylacetamide.

3.4. Effects of putative inhibitors and reductants

The inhibitory effect of various compounds on the activity of the purified native enzyme was examined (Table 3). D-Cycloserine did not influence the activity indicating that there is no carbonyl group involved in the reaction mechanism. The nitrilase was completely inactivated by thiol binding-reagent such as $AgNO_3$ and $CuSO_4$ showing that one or more SH-residue is essential for the enzyme activity. The enzyme was resistant to chelating reagents like EDTA and KCN, suggesting that the enzyme had no metal requirement. However, like the nitrilase from *Nocardia* sp. NCIB 11216 [18] the presence of both ferric and ferrous ions was found to decrease enzyme activity significantly. That might be due to redox effects on or complexing with the one or more essential thiol group. Conversely the enzyme was activated in the presence of the reductant 2-mercaptoethanol. 50 mM and 100 mM 2-mercaptoethanol resulted in 140% and 160% activity respectively. However, levels of more than 100 mM 2-mercaptoethanol did not lead to any higher enzyme activity.

The purified nitrilase was used to hydrolyse racemic 2-(methoxy)-mandelonitrile (Fig. 3). Due to the fact that α-hydroxy nitriles form an equilibrium with the equivalent aldehyde and HCN in aqueous solution, the amount of 2-(methoxy)-mandelonitrile decreased rapidly in the first few minutes, whilst the aldehyde concentration increased. At the same time 2-(methoxy)-mandelic acid was formed by the enzyme. A very low ($\leq 5\%$) but consistent

Table 2
Substrate specificity of nitrilase from *P. fluorescens* DSM 7155

Substrate	Relative activity [%]	K_m [mM]
Phenylacetonitrile	100	0.065
2-Phenylpropionitrile	2	–
2-Phenylbutyronitrile	0.1	–
2-(Methoxy)-mandelonitrile	10	0.087
Benzonitrile	0	–
2-Cyanotoluene	0	–
3-Cyanopyridine	0	–
3-Cyanobenzothiophene	0	–
Propionitrile	0	–
Butyronitrile	0	–
Valeronitrile	0	–

The reactions were carried out using the standard reaction mixture. The various nitriles (200 mM in methanol) were used as substrates in place of phenylacetonitrile. The synthesis of phenylacetic acid was taken as 100%. K_m-values were estimated from respective Lineweaver–Burk plots.

Table 3
Effects of various compounds on the activity of nitrilase from *P. fluorescens* DSM 7155

Addition	Conc. [mM]	Relative activity [%]
No addition control		100
$MnCl_2$	1	98
$FeCl_2$	1	82
	10	0
$FeCl_3$	1	1
$AgNO_3$	1	0
$CuSO_4$	1	0
D-Cycloserine	1	99
EDTA	1	95
KCN	1	99
	10	92
2-Mercaptoethanol	50	140
	100	160
	150	160

The enzyme was preincubated for 10 min with the various compounds to be tested, and then assayed in the standard reaction mixture without the addition of 2-mercaptoethanol.

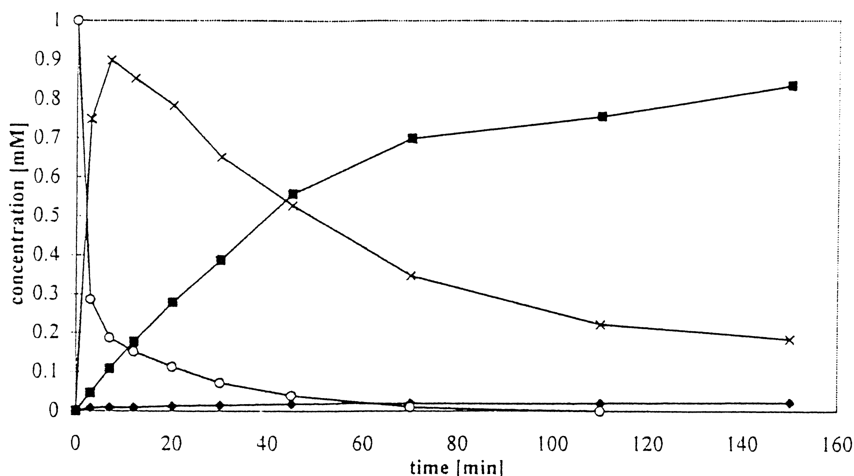

Fig. 3. Time course of 2-(methoxy)-mandelonitrile conversion by the nitrilase from *P. fluorescens* DSM 7155. The reaction mixture (1 ml) containing 1 μmol of 2-(methoxy)-mandelonitrile, 50 μmol Tris/HCl buffer (pH 7.4) and the enzyme (0.01 unit) was incubated at 30°C. 2-(methoxy)-mandelonitrile (○), 2-(methoxy)-mandelic acid (■), 2-(methoxy)-mandelic acid amide (♦) and 2-(methoxy)-benzaldehyde (x) were analysed as described in Section 2.

level of the equivalent amide was detected throughout the biotransformation. After 70 min nearly no nitrile was left in the reaction mixture, but the acid concentration continued to increase albeit less rapidly for a further 80 min. This observation, plus the concomitant progressive decrease in the aldehyde titre over the same time period, suggested that some of the aldehyde had chemically converted back to the nitrile which was then instantly hydrolysed by the nitrilase. After 40 min when the carboxylic acid product yield was approximately 50%, the extracted 2-(methoxy)-mandelic acid was almost exclusively the (*R*)-enantiomer (ee = 92%), whereas when the biotransformation was allowed to run to 150 min, the ee of the (*R*)-2-(methoxy)-mandelic acid had fallen to 27% at which point the reaction yield had risen to 85%.

4. Discussion

A nitrilase from *P. fluorescens* DSM 7155 was purified 259-fold with a yield of 10% from cell-free extract of the bacterium after growth on phenylacetonitrile as the sole nitrogen source. The presence of the nitrile compound was essential for the induction of nitrilase. The copresence of ammonium ions repressed the enzyme induction indicating some form of N-catabolite repression as observed with various other enzymes [19,20]. This suggests that the natural role of the nitrilase in this bacterium could be the provision of nitrogen from nitrile compounds.

Previously characterised nitrilases can be classified into three categories according to their substrate preferences. Aliphatic nitrilases which act exclusively on aliphatic nitriles such as the enzyme from *Rh. rhodochrous* K22 [21] or *Comomonas testosteroni* [17], aromatic nitrilases which act mainly on aromatic and heterocyclic nitriles like the enzymes found in *Arthrobacter* sp. or *Nocardia* sp. [7,18,22] and arylacetonitriles which act predominantly on arylacetonitriles, and which to date have been represented exclusively by enzymes present in strains of *Alcaligenes faecalis* [23,24]. Because the nitrilase from *P. fluorescens* DSM 7155 hydrolysed several arylacetonitriles, but no aliphatic, aromatic or heterocyclic nitriles, the enzyme is the first reported example of such an arylacetonitrilase present in a microorganism other than a species of *A. faecalis*.

Like the nitrilases from *Rh. rhodochrous* J1 [2] and *A. faecalis* JM3 [14], the enzyme re-

quired thiol reducing reagents to exhibit maximum activity. This finding, together with the strong inhibition of enzyme activity by thiol-complexing reagents suggests that one or more active thiol group plays an important role in the mechanism of this nitrilase. The essential role of a cysteine residue for activity of the equivalent nitrilase enzyme from *A. faecalis* JM3 as well as the nitrilases from *Rh. rhodochrous* K22 and *Arabidopsis thaliana* has been demonstrated by using site-directed mutagenesis [15,16,25]. Whereas the majority of all known nitrilases catalyse only the formation of carboxylic acid and ammonia from their nitrile substrates, the arylacetonitrilase from *P. fluorescens* DSM 7155 instead did form about 3–5% of the equivalent amide as a byproduct without being able to use it as an alternative substrate (e.g., see Fig. 3). Interestingly the nitrilases from a *Pseudomonas* strain which act on ricine nitrile and from *Fusarium oxysporum f.* sp. *melonis* which is active with various aromatic, heterocyclic and aliphatic nitriles, also accumulated a small amount of amide as a byproduct (4–6% and 9%, respectively) during nitrile hydrolysis [26,27].

The nitrilase from *P. fluorescens* DSM 7155 is probably an oligomer of the 40 kDa and 38 kDa subunits. Other oligomeric nitrilases with subunit molecular weights of 41–44 kDa have been isolated from various bacteria [15], including the well-studied commercially important strain *Rh. rhodocrous* J1 [2], although in most cases these enzymes are apparently composed of a single idiosynchratic subunit. One possibility that was considered was that the 38 kDa may have arisen from the larger 40 kDa protein by loss of a fragment during the purification procedure as was reported to be the relationship between the two different types of subunits isolated during purification of the nitrilase from *Acinetobacter* sp. AK 226 [28]. However, the limited sequence homology of the two subunits of the nitrilase purified from *P. fluorescens* DSM 7155 (62% for the N-terminal tridecyl peptides) suggested that for this particular enzyme, the two subunits represent discrete proteins: if so, then the native enzyme must exist as a heterologous copolymer.

The presence of a molecule of the CPN60 chaperonin (57 kDa) closely bound to the purified native nitrilase from *P. fluorescens* DSM 7155 was unexpected, and complicates the estimation of the molecular weight of the native nitrilase present in the bacterium. Taking into account the contribution made by the CPN60 protein, which is assumed to be serving some non-catalytic role associated with subunit assembly, the molecular weight of the most highly purified material isolated from the Superose 12 column (130 kDa) would suggest that the catalytic core of the nitrilase is a heterodimer with a combined molecular weight of 78 kDa. A similar conclusion was reached for the nitrilase from *Rh. rhodochrous* J1 [2]. However, it was subsequently reported [15] that when glycerol was added to the purified enzyme from *Rh. rhodochrous* J1, the dimeric form of the protein associates to form a dodecamer which retained nitrilase activity. The possibility that the nitrilase from *P. fluorescens* DSM 7155 may also exist as an oligomer of $n \times 78$ kDa is important as it may explain the presence of the CPN60 chaperonin protein identified as one of the three proteins found to constitute the most highly purified form of the enzyme. Chaperonins are known to bind to newly synthesised but incompletely assembled oligomeric enzymes [29]. If the nitrilase from *P. fluorescens* DSM 7155 is initially accumulated in cells as a heterodimer which only undergoes chaperonin-assisted assembly into a higher molecular weight complex at some later stage of the growth cycle, as has been reported for a cobalt-dependent nitrile hydratase enzyme from *Rh. rhodochrous* J1 [30], this could explain the presence of the CPN60 protein subunit in the nitrilase purified from cells of the bacterium harvested at the mid-exponential phase of growth.

The preferential formation of the (R)-enantiomer of 2-(methoxy)-mandelic acid (ee = 92% at approximately 50% yield) from the equiva-

lent racemic nitrile demonstrates that the nitrilase from DSM 7155 must hydrolyse one enantiomer of 2-(methoxy)-mandelonitrile more rapidly than the other. This conclusion is emphasised by the observation that by allowing the reaction to run on to 85% completion, the ee% of the recovered (R)-acid product fell substantially to only 27%. A comparison of the relative rate of hydrolysis of the two antipodes of the substrate is complicated by the fact that residual nitrile racemises spontaneously under the reaction conditions used. Clearly if the enzyme is to be used to yield chiral carboxylic acid end products of commercial interest, then careful monitoring of the progress of the biotransformation will be an essential prerequisite.

References

[1] Faber, K. Biotransformations in Organic Chemistry, 2nd edn., Springer-Verlag, Berlin, 1995.
[2] M. Kobayashi, T. Nagasawa, H. Yamada, Eur. J. Biochem. 182 (1989) 349.
[3] C.D. Mathew, T. Nagasawa, H. Yamada, Appl. Environ. Microbiol. 54 (1988) 1030.
[4] C. Bengis-Gerber, A.L. Gutman, Appl. Microbiol. Biotech. 32 (1989) 11.
[5] M. Kobayashi, T. Nagasawa, H Yamada, Appl. Microbiol. Biotech. 29 (1988) 231.
[6] T.C. Bhalla, A. Miura, A. Wakamoto, Y. Ohta, K. Furuhashi, Appl. Microbiol. Biotech. 37 (1992) 184.
[7] A.K. Bandyopadhyay, T. Nagasawa, Y. Asano, K. Fujishiro, Y. Tani, H. Yamada, Appl. Environ. Microbiol. 51 (1986) 302.
[8] M. Kobayashi, S. Shimizu, FEMS Microbiol. Lett. 120 (1994) 217.
[9] N. Layh, A. Stolz, S. Forster, F. Effenberger, H-J. Knackmuss, Arch. Microbiol. 158 (1992) 405.
[10] M. Phennig, K.D. Lippert, Arch. Microbiol. 55 (1966) 245.
[11] M. Bradford, Anal. Biochem. 72 (1976) 248.
[12] R. Gagnon, G. Grogan, S.M. Roberts, A. Willetts, J. Chem. Soc. Perkin Trans. 1 (1995) 1505.
[13] Grogan, G., PhD thesis, University of Exeter (1995).
[14] T. Nagasawa, J. Mauger, H Yamada, Eur. J. Biochem. 196 (1990) 765.
[15] M. Kobayashi, T. Nagasawa, H. Yamada, Trends Biotech. 10 (1992) 402.
[16] M. Kobayashi, H. Izui, T. Nagasawa, H. Yamada, Proc. Natl. Acad. Sci. 90 (1993) 247.
[17] S. Levy-Schil, J. Sikkema, A. Verheul, A. Bakker, J. Tramper, Gene 161 (1995) 15.
[18] D.B. Harper, Biochem. J. 165 (1977) 309.
[19] Martin, J.F., Aharononitz, Y., Antibiotics containing the *B*-lactam ring structure. Springer-Verlag, Berlin, 1983, p. 229.
[20] Y.-Q. Shen, J. Heim, J. Antiobiot. 37 (1984) 503.
[21] M. Kobayashi, N. Yanaka, T. Nagasawa, H. Yamada, J. Bacteriol. 172 (1990) 4807.
[22] D.B. Harper, Int. J. Biochem. 17 (1985) 677.
[23] J. Mauger, T. Nagasawa, H. Yamada, Arch. Microbiol. 155 (1990) 1.
[24] K. Yamamoto, Y. Ueno, K. Otsubo, H. Yamane, K-I. Komatsu, Y. Tani, J. Ferment. Bioeng. 73 (1992) 125.
[25] B. Bartel, G.R. Fink, Proc. Natl. Acad. Sci. 91 (1994) 6649.
[26] A. Goldlust, Z. Bohak, Biotechnol. Appl. Biochem. 11 (1989) 581.
[27] R.H. Hook, W.G. Robinson, J. Biol. Chem. 239 (1964) 4263.
[28] K. Yamamoto, K.I. Komatsu, Agric. Biol. Chem. 55 (1991) 1459.
[29] J.E. Rothman, Cell 59 (1989) 591.
[30] M. Kobayashi, H. Nishiya, T. Nagasawa, S. Horinouchi, T. Beppu, H. Yamada, Biochim. Biophys. Acta 1129 (1991) 23.
[31] D.M. Stalker, L.D. Malyj, K. McBride, Eur. J. Biol. Chem. 263 (1988) 6310.