Nitrilase-catalysed conversion of acrylonitrile by free and immobilized cells of *Streptomyces* sp.

V K Nigam[1,*], A K Khandelwal[1], R K Gothwal[1], M K Mohan[1], B Choudhury[2], A S Vidyarthi[3] and P Ghosh[1]

[1]*Birla Institute of Scientific Research, Statue Circle, Jaipur 302 001, India*
[2]*Faculty of Biological Science, Indian Institute of Technology, Roorkee 247 667, India*
[3]*Department of Biotechnology, Birla Institute of Technology, Mesra, Ranchi 835 215, India*

**Corresponding author (Fax, 91-141-2385121; Email, nigam.bisr@gmail.com)*

The biotransformation of acrylonitrile was investigated using thermophilic nitrilase produced from a new isolate *Streptomyces* sp. MTCC 7546 in both the free and immobilized state. Under optimal conditions, the enzyme converts nitriles to acids without the formation of amides. The whole cells of the isolate were immobilized in agar-agar and the beads so formed were evaluated for 25 cycles at 50°C. The enzyme showed a little loss of activity during reuse. Seventy-one per cent of 0.5 M acrylonitrile was converted to acid at 6 h of incubation at a very low density of immobilized cells, while 100% conversion was observed at 3 h by free cells.

[Nigam V K, Khandelwal A K, Gothwal R K, Mohan M K, Choudhury B, Vidyarthi A S and Ghosh P 2009 Nitrilase-catalysed conversion of acrylonitrile by free and immobilized cells of *Streptomyces* sp.; *J. Biosci.* **34** 21–26]

1. Introduction

Nitrilases that hydrolyse organic nitriles to carboxylic acids and ammonia are a commercially interesting group of enzymes, as nitriles are important intermediates in the chemical synthesis of various products. Nitriles are also a major potential source of environmental hazards (Kobayashi *et al* 1998; Nagasawa *et al* 2000; Brandao and Bull 2003). Nitrile compounds are generally catabolised by microorganisms directly to carboxylic acids and ammonia using a nitrilase or nitrile hydratase that forms an amide as an intermediate degradation product, followed by amidase that converts the amide to the corresponding carboxylic acid and ammonia. Nitrile-converting biocatalysts have considerable industrial interest as they can be used to treat toxic nitrile- and cyanide-containing wastes and as agents for the synthesis of chemicals that have widespread applications (Heald *et al* 2001; Trott *et al* 2001).

These enzymes also find application in preparative organic chemistry because of their capacity to convert readily available nitriles into the corresponding higher-value amides and acids under mild conditions without altering other labile reactive groups and, in some cases, with enantio- or regio-selective properties (Yamamoto *et al* 1991; Kaul *et al* 2004; Banerjee *et al* 2006).

Various nitrilases that hydrolyse aromatic and aliphatic substrates with different properties have been described from a variety of microorganisms such as *Rhodococcus*, *Acinetobacter*, *Pseudomonas*, *Acidovorax, Streptomyces*, etc. (Mauger *et al* 1990; Nagasawa *et al* 1990; Gavagan *et al* 1999; Rezende *et al* 2003; Komeda *et al* 2004; Khandelwal *et al* 2007).

Mesophilic nitrilases are relatively unstable at higher temperatures. Hence, interest in enzymes from thermophilic organisms has been greatly stimulated by the fact that these proteins are resistant to chemical and physical denaturation and to proteolysis (Cowan *et al* 1998; Almatawah *et al* 1999). The recent expansion of nitrile-dependent biotransformation and enzymatic detoxification of nitrile-based herbicides, and the instability of mesophilic nitrile-metabolizing enzymes have led to the investigation of thermophilic microbes as an alternative source of biotransformation (Bunch 1998; Tauber

Keywords. Aliphatic nitriles; biotransformation; immobilization; nitrilase; *Streptomyces*; thermophilic

et al 2000). Biotransformation has become a new tool for the modification of biotechnology and various processes, and is being studied as a substitute for most of the chemical processes. The immobilization of microbial cells involved in the production of useful compounds is now gaining more importance than immobilized enzymes because it eliminates the need for release of intracellular enzymes and the succeeding purification steps. Immobilization provides a way to maintain high cell densities and high flow rates during continuous processing. In addition, immobilized cells are reusable.

There are very few reports on the immobilization of nitrilases. We therefore attempted to immobilize whole cells of *Streptomyces* sp. MTCC 7546 which produce thermostable nitrilase in a cheaper matrix (agar-agar) by entrapment, and investigated the biotransformation of acrylonitrile. We also compared the efficiency of biotransformation between free and entrapped cells.

2. Materials and methods

2.1 *Growth conditions*

Streptomyces sp. MTCC 7546, a new nitrilase-producing isolate, was routinely grown in mineral base media modified to include yeast extracts (1 g/l), glycerol (10 g/l), trisodium citrate (0.2 g/l), benzonitrile (10 mM); 5X mineral base (5 g/l NaCl, 1.35 g/l KH_2PO_4, 1 g/l $MgSO_4.7H_2O$, 0.87 g/l K_2HPO_4, 0.05 g/l $CaCl_2$, 1.25 mg /l $FeCl_3$) and trace element solution (0.3 g/l H_3BO_3, 0.2 g/l $CoCl_2.6H_2O$, 0.1 g/l $ZnSO_4.7H_2O$, 0.03 g/l $MnCl_2.4H_2O$, 0.03 g/l $Na_2MoO_4.H_2O$, 0.02 g/l $NiCl_2.6H_2O$, and 0.01 g/l $CuCl_2.2H_2O$). Benzonitrile and trace element solution were added to the medium after sterilization.

The culture was maintained on Luria–Bertani (LB) medium supplemented with 10 mM adiponitrile. All the chemicals used during the experiments were of analytical grade and obtained from standard companies.

The liquid media were inoculated from a freshly cultured slant/petriplate and incubated for 24 h at 45°C in an orbital shaker set at 200 rpm. Well-grown cells from the enzyme production medium were harvested and centrifuged at 10 000 rpm for 15 min at 4°C. The cell pellet was washed twice with 0.1 M phosphate buffer (pH 7.4) containing 5 mM EDTA dissolved in a suitable amount of the same buffer and used for immobilization with agar-agar powder as the carrier. Cell growth was estimated turbidimetrically at 600 nm.

2.2 *Immobilization*

A definite quantity of agar-agar (Hi-media, Mumbai, India) was dissolved in an appropriate amount of buffer and autoclaved. The cell suspension was mixed aseptically with the agar at 40–50°C and poured on a flat-bottom petriplate (90 mm × 15 mm) to get a final concentration of 2% and 3% agar, respectively. The agar plate was kept at room temperature for solidification and finally the agar block was cut using a sterile borer 4 mm in diameter. The agar beads so formed were washed with phosphate buffer and sterile distilled water twice and finally stored at 4°C till further use (Adinarayana *et al* 2005; Nigam *et al* 2005). The wet weight of the cells in each bead was maintained at approximately 0.0054 g, which is equivalent to a dry cell weight of 0.001 g. One to two beads were used in 1.0 ml of reaction mixture with different concentrations of acrylonitrile and the biotransformation efficiency was evaluated. The immobilized whole cell (one bead) was also tested for its reusability over 25 cycles at 50°C.

2.3 *Enzyme assay*

The activity of nitrilase was studied at 50°C for 15 min in a 1.0 ml reaction mixture that contained 0.1 M potassium phosphate buffer with 5 mM EDTA (pH 7.4), acrylonitrile (10 mM unless stated otherwise) and 0.1 ml whole cell suspension (~0.0010 g dry cell weight). The reaction was stopped by adding 0.05 ml 2N HCl and the mixture centrifuged at 10 000 rpm for 10 min at 4°C. The ammonia released during the incubation was measured by Bertholet reaction (Piotrowski *et al* 2001). Enzyme activity is expressed in IU, which is defined as μg of ammonia released/min/g of dry cell weight.

The analysis of acrylic acid and acrylamide was performed by Shimadzu HPLC using a CLC-ODS (Shim-pack, Schimadzu, Japan) column. The mobile phase comprises 10 mM phosphate buffer (pH 2.1) containing 4% (v/v) acetonitrile at a flow rate of 1 ml/min. The product was determined by a UV detector at a wavelength of 210 nm.

The utilization of acrylonitrile during the enzymatic reaction was measured by gas chromatography (Nucon-5765, Aimil Instruments, India) using a packed bed Porapack Q column (200 x 0.2 cm) equipped with a flame ionization detector (FID) in isocratic condition. The temperature of the oven was 140°C, while the temperature of both the injector and the detector was kept at 210°C. The flow of N_2, H_2 and air gases was maintained at 40 ml/min, 60 ml/min and 40 ml/min, respectively. Of the reaction mixture, 5 μl was injected and the residual acrylonitrile concentration was calculated against the standard (Khandelwal *et al* 2007).

3. Results

Immobilized biocatalysts such as enzymes, microorganisms, organelles, and plant and animal cells have added new

dimensions to the application potential of the rapidly advancing field of biotechnology. Immobilized whole cells have been well investigated as they offer numerous advantages over suspended/free cells. We used whole cells of a thermostable nitrilase-producing culture of *Streptomyces* sp. MTCC 7546 isolated from a soil sample in the presence of adiponitrile as an inducer. The cells were immobilized in agar-agar and the biotransformation of acrylonitrile was investigated. Immobilization of cells was studied in different concentrations of agar; initial studies of enzymatic activity revealed that a final concentration of 2% was best for the entrapment of whole cells. When the number of beads was varied for the biotransformation of acrylonitrile (0.2 M) it was observed that 2–3 beads per reaction gave the maximum conversion (data not shown).

3.1 *Biotransformation of acrylonitrile using free cells*

Two different concentrations of acrylonitrile (0.1 M and 0.5 M) were evaluated for their conversion to acid at optimal conditions as stated in the Materials and methods section. The time of incubation of the enzyme substrate reaction mixture was also varied during the course of the investigation. The results are shown in table 1. It was observed that up to 85% of acrylonitrile was converted by the enzyme at 15 min when 0.1 M acrylonitrile was used as the substrate. With 0.5 M concentration, almost 100% conversion was noticed at 3 h of incubation. This type of study has not been undertaken often in the nitrilase system and such a high concentration of substrate conversion has rarely been observed.

3.2 *Biotransformation of acrylonitrile using immobilized cells*

The conversion of acrylonitrile was observed by varying the number of beads, acrylonitrile concentrations and time of incubation. The results are shown in figures 1 and 2. It was observed that up to 96% of acrylonitrile was converted to acrylic acid using one bead in the reaction mixture (0.1 M concentration of acrylonitrile), while in the case of the 0.5 M substrate, approximately 43% of residual acrylonitrile was observed at 6 h of incubation (figure 1). Using two beads per reaction mixture, the biotransformation efficiency increased and only 29% of residual acrylonitrile was found after 6 h of incubation at an acrylonitrile concentration of 0.5 M (figure 2). The efficiency of bioconversion of different concentrations of acrylonitrile using immobilized whole cells is also represented in table 1. The substrate consumption profile using different modes of cells with respect to the substrate blank (control) was calculated by gas chromatography and is shown in table 2. It is clear from the table that the peak area obtained with free cells against different concentrations of the substrate is very low as compared with immobilized beads, indicating that free cells had the highest nitrilase activity.

The activity of immobilized whole cells is less than that of free cells and this can be explained on the basis of mass

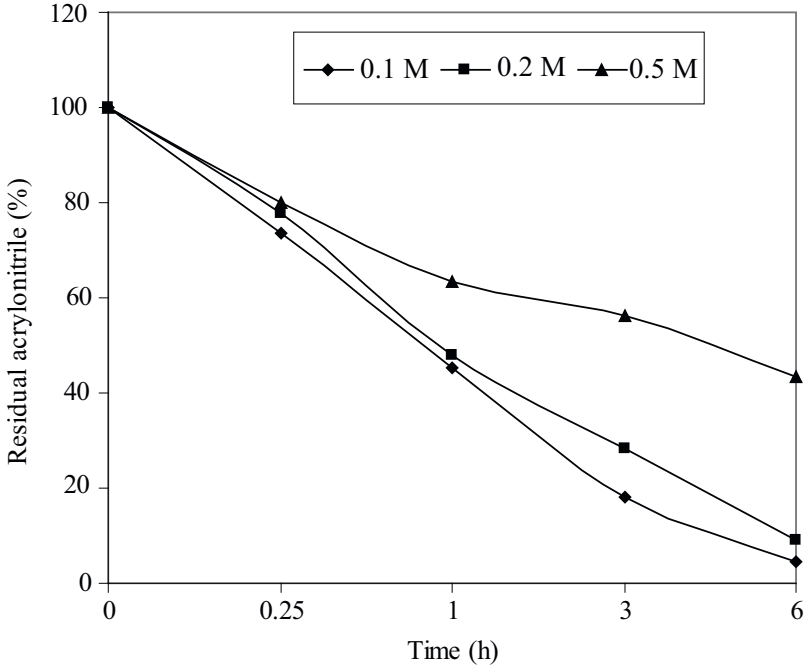

Figure 1. Biotransformation of acrylonitrile with one bead.

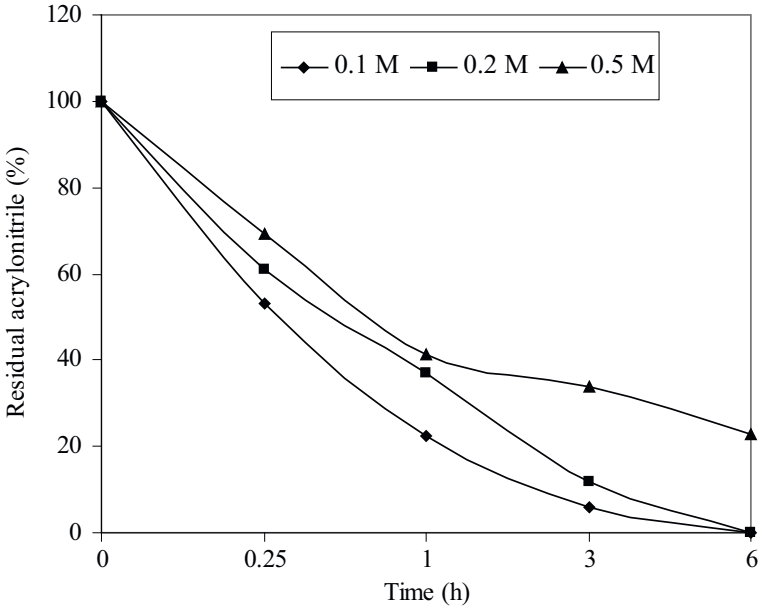

Figure 2. Biotransformation of acrylonitrile with two beads.

Table 1. Biotransformation of acrylonitrile with free and immobilized whole cells

	Residual acrylonitrile (%)								
	Free cells			One bead			Two beads		
Time (h)	0.1 M	0.2 M	0.5 M	0.1 M	0.2 M	0.5 M	0.1 M	0.2 M	0.5 M
0.25	15.51	Not done	37.17	73.46	77.69	80.10	53.29	60.86	69.93
1	Nil	Not done	2.07	45.14	47.74	63.21	22.53	36.90	41.49
3	Nil	Not done	0.05	18.3	28.12	56.04	5.94	11.86	34.00
6	Nil	Not done	Nil	4.58	9.24	43.44	0.00	0.00	22.70

Table 2. Peak areas of biotransformation of acrylonitrile by gas chromatographic analysis with free and immobilized whole cells

	Peak area (millivolt)											
	Control (Substrate blank)			Free cells			One bead			Two beads		
Time (h)	0.1 M	0.2 M	0.5 M	0.1 M	0.2 M	0.5 M	0.1 M	0.2 M	0.5 M	0.1 M	0.2 M	0.5 M
0.25	10 660 986	20 539 881	50 745 695	1 653 519	Not done	18 862 174	78 315 603	15 957 433	40 647 301	5 681 239	1 250 0571	35 486 464
1	10 522 861	20 346 589	50 525 833	0.00	Not done	1 045 885	47 500 194	9 713 462	31 937 379	2 370 801	7 507 891	2 096 3168
3	10 379 161	20 196 563	50 403 669	0.00	Not done	25 202	1 899 3864	5 679 273	28 246 216	616 522	2 395 312	1 713 7247
6	10 174 568	19 855 644	50 279 276	0.00	Not done	0.00	465 995	18 346 61	21 841 317	0.00	0.00	11 413 395

transfer limitation of substrate to the immobilized cells. The biotransformation of acrylonitrile was found to be 82%, 72% and 44% of free cells with one bead per reaction mixture at acrylonitrile concentrations of 0.1 M, 0.2 M and 0.5 M, respectively, when incubated for 3 h.

3.3 *Reusability of immobilized cells*

In order to evaluate the reusability of immobilized whole cells, the biotransformation of acrylonitrile was studied at optimal conditions for 25 cycles using 0.1 M acrylonitrile

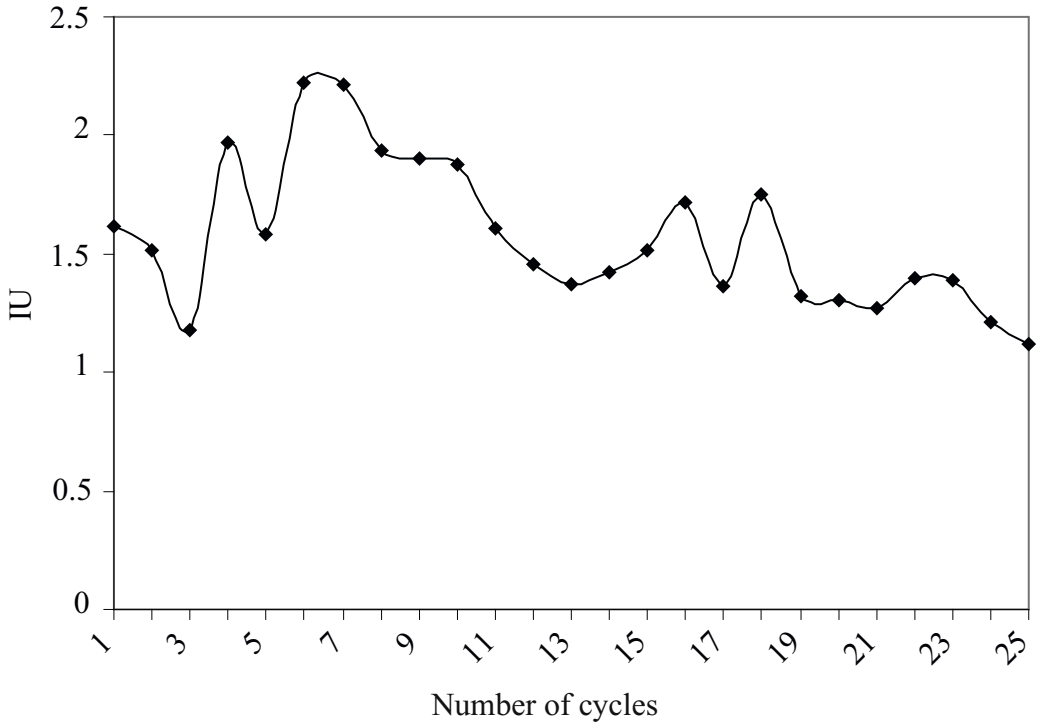

Figure 3. Reusability of immobilized cells (one bead).

as the substrate and one bead per reaction mixture. Each biotransformation cycle was of 2 h duration. After each cycle the bead was washed with sterile water and reused. The results are presented in figure 3.

Figure 3 reveals that activity with immobilized cells is stable up to almost 20 cycles without much loss. Therefore, immobilized cells can efficiently be used for the bioconversion of acrylonitrile to acrylic acid. It is also observed that approximately 35% activity is lost after 25 cycles have been operated.

4. Discussion

Nitrile-metabolizing enzymes have the potential to perform valuable biotransformations such as the production of acids and amides of industrial importance. The chemical conversion of nitriles to acids and amides is feasible but requires relatively harsh conditions such as heat, acid or alkali. Most of the nitrile-metabolizing enzymes (nitrilase or nitrile hydratase/amidase) have been explored from mesophilic sources, especially from the rhodococcal system, and are widely used for the production of acrylamide, acrylic acid, nicotinamide, etc. Some of them are also involved in enantio- and regio-specific biotransformation. A thorough literature review was performed and it was observed that though a number of strains have been used for the production of nitrilase, very little emphasis has been given on the immobilization of nitrilase-producing isolates. The bioconversion of benzonitrile to benzoic acid using immobilized cells of *Nocardia globerula* NHB-2 was reported by Raj *et al* (2007). They observed 100% enzymatic activity at 30°C and a loss of 50% activity at 40°C. Only one thermophilic microorganism, *Bacillus* sp. UG-5B, has been reported to biotransform benzonitrile in an immobilized state with retention of 100% activity after eight reaction cycles (Kabaivanova *et al* 2005). We have described the immobilization of whole cells of *Streptomyces* sp. MTCC 7546 in a cheaper matrix and evaluated the applicability of whole cells as well as immobilized cells for the biotransformation of acrylonitrile at a high temperature (50°C) in a batch mode of operation. The operational stability of the immobilized cells showed that they could be reused for 25 cycles with a loss in enzymatic activity of approximately 20%.

5. Conclusion

Nitrilase produced from *Streptomyces* sp. MTCC 7546 catalyses the hydrolysis of nitriles to acids without the formation of amides. The use of biological systems to convert nitrile-containing substrates to carboxylic acids is an attractive alternative to chemical methods. The whole

cells of the bacteria were immobilized in agar powder and evaluated for their enzymatic activity at 50°C. The biotransformation of acrylonitrile using immobilized cells is superior to that using suspension cells as the long life-term stability, reusability and possibility of scale-up is greater. A bioremediation process could be conducted efficiently using immobilized cells with nitrilase activity.

Acknowledgements

One of the authors (AKK) wishes to acknowledge the financial assistance received from Indian Council of Medical Research, New Delhi, for carrying out this work.

References

Almatawah Q A, Cramp R and Cowan D A 1999 Characterization of an inducible nitrilase from a thermophilic bacillus; *Extremophiles* **3** 283–291

Adinarayana K, Jyothi B and Ellaiah P 2005 Production of alkaline protease with immobilized cells of *B. subtilis* PE-11 in various matrices by entrapment technique; *AAPS PharmSCiTech.* **6** 391–397

Banerjee A, Kaul P and Banerjee U C 2006 Purification and characterization of an enantioselective arylacetonitrilase from *Pseudomonas putida*; *Arch. Microbiol*. **184** 407–418

Brandao P F B and Bull A T 2003 Nitrile hydrolyzing activities of deep-sea and terrestrial mycolate actinomycetes; *Antonie van Leeuwenhoek* **84** 89–98

Bunch A W 1998 Biotransformation of nitriles by rhodococci; *Antonie van Leeuwenhoek* **74** 89–97

Cowan D, Cramp R, Pereira R, Graham D and Almatawah Q 1998 Biochemistry and biotechnology of mesophilic and thermophilic nitrile metabolizing enzymes; *Extremophiles* **2** 207–216

Gavagan J E, DiCosimo R, Eisenberg A, Fager S K, Folsom P W, Hann E C, Schneider K J and Fallon R D 1999 A Gram-negative bacterium producing a heat-stable nitrilase highly active on aliphatic dinitriles; *Appl. Microbiol. Biotechnol*. **52** 654–659

Heald S C , Brandao P F B, Hardicre R and Bull A T 2001 Physiology, biochemistry and taxonomy of deep-sea nitrile metabolizing *Rhodococcus* strains; *Antonie van Leeuwenhoek* **80** 169–183

Kabaivanova L, Dobreva E, Dimitrov P and Emanuilova E 2005 Immobilization of cells with nitrilase activity from a thermophilic bacterial strain; *J. Ind. Microbiol. Biotechnol*. **32** 7–11

Kaul P, Banerjee A, Mayilraj S and Banerjee U C 2004 Screening for enantioselective nitrilase: kinetic resolution of racemic mandelonitrile to (R)-(-)- mandelic acid by new bacterial isolates; *Tetrahedron: Asymmetry* **15** 207–211

Khandelwal A K, Nigam V K, Choudhury B, Mohan M K and Ghosh P 2007 Optimization of nitrilase production from a new thermophilic isolate; *J. Chem. Technol. Biotechnol*. **82** 646–651

Kobayashi M, Nagasawa T and Yamada H 1998 Regio-specific hydrolysis of dinitrile compounds by nitrilase from *Rhodococcus rhodochrous* J1; *Appl. Microbiol. Biotechnol*. **29** 231–233

Komeda H, Harada H, Washika S, Sakamoto T, Ueda M and Asano Y A 2004 Novel R- stereo selective amidase from *Pseudomonas* sp. MCI3434 acting on piperazin-2-tert- butylcarboxamide; *Eur. J. Biochem*. **271** 1580–1590

Mauger J, Nagasawa T and Yamada H 1990 Occurrence of a novel nitrilase arylacetonitrilase, in *Alcaligenes faecalis* JM3; *Arch. Microbiol.* **155** 1–6

Nagasawa T, Nakamura T and Yamada H 1990 Production of acrylic acid and methacrylic acid using *Rhodococcus rhodochrous* J1 nitrilase; *Appl. Microbiol. Biotechnol*. **34** 322–324

Nagasawa T, Wieser M, Nakamura T, Iwahara H, Yoshida T and Gekko K 2000 Nitrilase of *Rhodococcus rhodochrous* J1; conversion into the active form by subunit association; *Eur. J. Biochem*. **267** 138–144

Nigam V K, Kundu S and Ghosh P 2005 Single step conversion of cephalosporin-C to 7-ACA by free and immobilized cells of *P. diminuta*; *Appl. Biochem. Biotechnol*. **126** 13–21

Piotrowski M, Schonfelder S and Weiler E W 2001 The *Arabidopsis thaliana* isogene *NIT4* and its orthologs in tobacco encode *β*-cyano-L-alanine hydratase/nitrilase; *J. Biol. Chem*. **276** 2616–2621

Raj J, Singh N, Prasad S, Seth A and Bhalla T C 2007 Bioconversion of benzonitrile to benzoic acid using free and entrapped cells of *Nocardia globerula* NHB-2; *Acta Microbiol. Immunol. Hung.* **54** 79–88

Rezende R P, Dias J C T, Monteiro A S, Carraza F and Linardi V R 2003 The use of acetonitrile as the sole nitrogen and carbon source by *Geotrichum* sp. JR1; *Braz. J. Microbiol.* **34** 117–120

Tauber M M, Cavaco-Paulo A, Robra K H and Gubitz G M 2000 Nitrile hydratase and amidase from *Rhodococcus rhodochrous* hydrolyze acrylic fibers and granular polyacrylonitriles; *Appl. Environ. Microbiol*. **66** 1634–1638

Trott S, Bauer R, Knackmuss H J and Stolz A 2001 Genetic and biochemical characterization of an enantioselective amidase from *Agrobacterium tumifaciens* strain d3; *Microbiology* **147** 1815–1824

Yamamoto K, Oishi K, Fujimatsu I and Komatsu K I 1991 Production of (R)-(-)- mandelic acid from mandelonitrile by *Alcaligenes faecalis* ATCC 8750; *Appl. Environ. Microbiol*. **57** 3028–3032

MS received 16 June 2008; accepted 16 October 2008

*e*Publication: 21 January 2009

Corresponding editor: Dipankar Chatterji