Enhancing the catalytic potential of nitrilase from Pseudomonas putida for stereoselective nitrile hydrolysis

ARTICLE *in* APPLIED MICROBIOLOGY AND BIOTECHNOLOGY · SEPTEMBER 2006

Impact Factor: 3.34 · DOI: 10.1007/s00253-005-0255-8 · Source: PubMed

3 AUTHORS, INCLUDING:

Anirban Banerjee
Indian Institute of Technology Bombay
26 PUBLICATIONS **1,419** CITATIONS

SEE PROFILE

Appl Microbiol Biotechnol (2006) 72: 77–87
DOI 10.1007/s00253-005-0255-8

Anirban Banerjee · Praveen Kaul
U. C. Banerjee

Enhancing the catalytic potential of nitrilase from *Pseudomonas putida* for stereoselective nitrile hydrolysis

Received: 2 October 2005 / Revised: 2 October 2005 / Accepted: 5 November 2005 / Published online: 4 January 2006

Abstract (*R*)-mandelic acid was produced from racemic mandelonitrile using free and immobilized cells of *Pseudomonas putida* MTCC 5110 harbouring a stereoselective nitrilase. In addition to the optimization of culture conditions and medium components, an inducer feeding approach is suggested to achieve enhanced enzyme production and therefore higher degree of conversion of mandelonitrile. The relationship between cell growth periodicity and enzyme accumulation was also studied, and the addition of the inducer was delayed by 6 h to achieve maximum nitrilase activity. The nitrilase expression was also authenticated by the sodium dodecyl phosphate-polyacrylamide gel electrophoresis analysis. *P. putida* MTCC 5110 cells were further immobilized in calcium alginate, and the immobilized biocatalyst preparation was used for the enantioselective hydrolysis of mandelonitrile. The immobilized system was characterized based on the Thiele modulus (ϕ). Efficient biocatalyst recycling was achieved as a result of immobilization with immobilized cells exhibiting 88% conversion even after 20 batch recycles. Finally, a fed batch reaction was set up on a preparative scale to produce 1.95 g of (*R*)-(-)-mandelic acid with an enantiomeric excess of 98.8%.

Introduction

Nitrile compounds are convenient and synthetically important chemical building blocks because they are readily generated and can be converted into high-value acids and amides (Sugai et al. 1997). There is a considerable industrial interest in enzymatic conversion of nitriles owing to the desirability of conducting such conversions under mild conditions that would not alter other labile reactive groups. From this point of view, nitrile hydrolases are expected to gain immense importance as useful biocatalyst for organic chemical processing (Mylerova and Martinkova 2003). This environmental friendly bioconversion allows clean and mild synthesis with high selectivity and yield. Nitrilases (EC 3.5.5.1) are an important class of nitrilase superfamily (Brenner 2002) that converts nitriles to corresponding carboxylic acids and ammonia. Microorganism may also follow a bienzymatic pathway comprising nitrile hydratases (NHases; EC 4.2.1.84) and amidases (EC 3.5.14) for the transformation of nitriles in which the nitrile is first converted to an amide by NHases, followed by its conversion to acids by amidases. To date, many nitrilases, NHases and amidases were reported from a variety of microorganisms (Banerjee et al. 2002; O'Reilly and Turner 2003). In spite of the great synthetic potential that nitrilases have to offer, their utilization as a versatile biocatalyst is largely unexploited. This could be attributed to their unstable nature and lack of ready availability. Ready availability of stabilized nitrilase preparations would give access to wider synthetic application of this class of enzymes.

Arylacetic acids are known to be important pharmaceutical intermediates. A practically feasible route for the production of such acids in enantiomerically pure form is the hydrolysis of the corresponding nitriles. There are very few reports on stereoselective arylacetonitrilases applied in the conversion of arylacetonitriles (Kakeya et al. 1991; Yamamoto et al. 1991; Layh et al. 1992). Recently, we have isolated some new bacterial strains using an 'intelligent' screening approach (Banerjee et al. 2003), which convert racemic mandelonitrile to (*R*)-(-)-mandelic acid, an important chiral building block, with high enantiomeric excess (Fig. 1). Among them, *Pseudomonas putida* MTCC 5110 showed comparatively high yield with excellent enantiomeric excess (ee; %) in shortest possible time (Kaul et al. 2004). The nitrilase of this organism was also found to be highly stable under operational conditions.

A. Banerjee · P. Kaul ·
U. C. Banerjee (✉)
Department of Pharmaceutical Technology (Biotechnology),
National Institute of Pharmaceutical Education
and Research Sector-67,
SAS Nagar,
160 062 Punjab, India
e-mail: ucbanerjee@niper.ac.in
Tel.: +91-172-2214682
Fax: +91-172-2214692

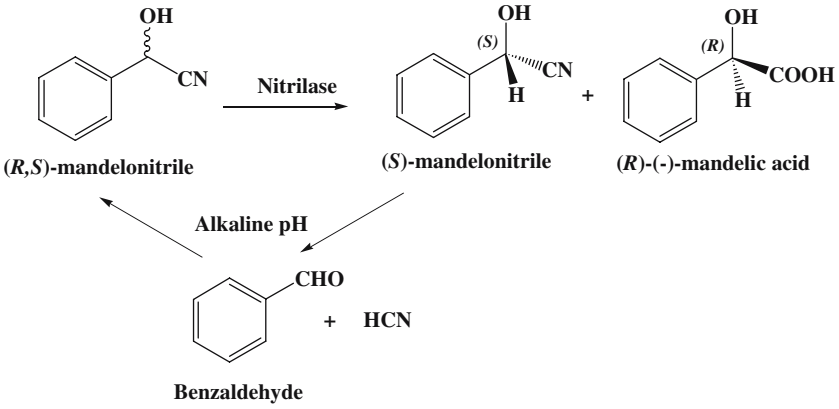

Fig. 1 Enantioselective hydrolysis of manelonitrile to (*R*)-(-)-mandelic acid by nitrilase from *Pseudomonas putida* MTCC 5110

These interesting results have prompted us to investigate the optimum culture conditions for the production of nitrilase by *P. putida* and utilize the cells produced thereof for further biocatalytic reaction. Variation in culture conditions plays an important role in the formation of desirable enzymes, and several such reports exist in which optimization of physico-chemical parameters resulted in enhancement of nitrile hydrolyzing activity (Yamada et al. 1986; Watanabe et al. 1987; Nagasawa et al. 1988, 1990). In a special case, variation in cultivation condition also resulted in the increased enantioselectivity of the enzyme (Wu and Li 2002). In this manuscript, we report a detailed study on the effect of different physico-chemical parameters and nutritional requirements for the production of enantioselective arylacetonitrilase by *P. putida* MTCC 5110. Different reaction engineering aspects of the nitrile hydrolysis with both free and immobilized cell preparations were also investigated.

Materials and methods

Chemicals

Racemic mandelonitrile, acetonitrile (ACN), sodium alginate, (*R*)-mandelic acid and (*S*)-mandelic acid were obtained from Aldrich Chemical Company (Milwaukee, USA). Different media components were obtained from Hi-Media Inc. (Mumbai, India). All other chemicals used were of analytical grade and procured from standard companies.

Microorganism and cultivation conditions

P. putida, isolated from soil in our laboratory, was used in this study (Kaul et al. 2004). The microorganism was deposited at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India and given the accession number MTCC 5110. The microorganism was grown aerobically at 30°C for 12 h in a basal media with the following composition (in g l^{-1}): peptic digest of animal tissue, 5; NaCl, 5; and yeast extract, 3. This inoculum (2% v/v) was transferred to the same medium containing isobutyronitrile (1 g l^{-1}l) as inducer, and the cells were further grown. The biomass produced was determined by measuring the $A_{600\ nm}$ using a UV–Vis Spectrophotometer (Beckman DU 7400, USA). Cells were harvested by centrifugation (10,000×*g*, 10 min, 4°C) at regular intervals. The harvested cells were washed with Tris–HCl buffer (0.1 M, pH 7) and resuspended in the same buffer to give an appropriate cell concentration.

Immobilization of *P. putida* cells in alginate beads

After optimization of medium components and physico-chemical parameters, the microorganism was grown in the optimized medium comprising of the following (in g l^{-1}l): starch, 5; soy peptone, 5; sodium chloride, 5; yeast extract, 1.5; malt extract, 1.5 (pH 7.5) at 30°C. Two percent (v/v) of this inoculum was transferred to the same medium containing ACN (0.5 g l^{-1}l) as inducer, and the cells were grown for further 12 h. The cell suspension obtained after suspending the cells in buffer was then added to sodium alginate solution, and the slurry (containing 3%, w/v Na-alginate) was added dropwise to an ice-cold solution of calcium chloride (2%, w/v) under constant stirring. After stirring for 2 h, the solution was decanted, and the resulting beads were stored in fresh calcium chloride solution until use. Before use, the beads were washed with Tris–HCl buffer (0.1 M, pH 7) to remove excess $CaCl_2$ and finally suspended in the same buffer for further biocatalytic reaction.

Enzyme assay

Mandelonitrile hydrolyzing activity was assayed in a reaction mixture (10 ml) containing free or immobilized cells of *P. putida* MTCC 5110 suspended in Tris–HCl buffer (0.1 M, pH 7) at a final concentration of 50 mg ml^{-1} and racemic mandelonitrile (15 mM). The reaction mixture was shaken for 20 min at 35°C and then stopped by addition of 1 N HCl (0.3 ml). Amount of mandelic acid formed and the degree of mandelonitrile conversion were

determined by Reversed phase high-performance liquid chromatography (RP-HPLC). One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μmol of mandelic acid per minute under standard assay conditions.

Isolation of (*R*)-(-)-mandelic acid from the reaction mixture

After cells/aliginate beads were removed from the reaction mixture by centrifugation (15,000×*g*, 10 min, 4°C), the pH of the reaction mixture was adjusted to 8.5 with 2 N NaOH and washed with equal volume of ether. The pH of the aqueous layer was then readjusted to 1.5 with 6 N HCl, and the desired product was extracted with equal volume of ether. The extract was concentrated under reduced pressure to yield mandelic acid.

Analytical methods

High-performance liquid chromatography

The amounts of mandelic acid, mandelamide and mandelonitrile were assayed by analytical high-performance liquid chromatography (HPLC; model 10AD VP; (Shimadzu, Japan) equipped with a LiChroCART RP-18 column (250×4 mm, 5 μm) (Merck, Germany) at a flow rate of 0.8 ml min^{-1}, with a solvent system of 0.01 M phosphate buffer (pH 4.8) and methanol (65:35, v/v). The retention times for mandelic acid and mandelonitrile were 2.6 and 18.3 min, respectively. $A_{254\ nm}$ was measured. Detection range for mandelic acid is 100–1000 μg ml^{-1} and that for mandelonitrile is 10–100 μg ml^{-1}.

The optical purity of mandelic acid was determined by analysis of the enantiomers on CHIRALCEL-OD-H column (250×0.46 mm, 5 μm; Daicel Chemical Industries, USA) at a flow rate of 0.5 ml min^{-1}, with a mobile phase containing hexane, isopropanol and trifluoroacetic acid (90:10:0.2, v/v). The retention times for (*S*)-(+)- and (*R*)-(-)-isomers were 15.5 and 17.5 min, respectively. $A_{254\ nm}$ was measured.

Preparation of cell-free extracts

Washed cells were suspended in Tris–HCl buffer (0.1 M, pH 7.5) containing 1 mM dithiothreitol and then disrupted using a high-pressure homogenizer (Thermo Spectronic, Rochester, USA) at 16×10^3 psi, 2 cycles, 1 ml min^{-1} flow rate. The cell debris was removed by centrifugation (20,000×*g*, 30 min, 4 C), and the supernatant obtained was designated as cell-free extract (CFE).

Protein determination

Protein was determined by the Bradford method (Bradford 1976) with bovine serum albumin as standard (10–100 μg ml^{-1}).

Gel electrophoresis

Gel electrophoresis was performed in 12% sodium dodecyl phosphate (SDS)-polyacrylamide gels with a Tris–Glycine buffer system (Lammeli 1970), and the gel was stained with Commassie brilliant blue (R 250). Quantification of nitrilase in gel slab after separation by elctrophoresis was carried out with Gel Doc 2000 and QuantityOne software (Bio-Rad Laboratories, Hercules, USA).

Results

Optimization of physico-chemical parameters and medium components

Effect of environmental factors

The effect of physico-chemical parameters on the biomass and nitrilase production was studied in the basal media (see "Materials and methods"). Maximum nitrilase activity was obtained when the initial pH of the medium was adjusted to 7.5 (specific activity 1.54 μmol min^{-1} g, biomass 4.59 g l^{-1}), although highest biomass was obtained at pH 8.0 (specific activity 1.24 μmol min^{-1} g, biomass 5.02 g l^{-1}). Sharp drop in the biomass production as well as in specific activity and conversion of mandelonitrile was observed when the initial pH of the medium was adjusted above 8.5 (specific activity 0.84 μmol min^{-1} g, biomass 2.89 g l^{-1}). Production studies were also carried out at different temperatures ranging from 20 to 40°C to determine the effect of temperature on nitrilase production. The organism was found to grow maximally at 25°C (specific activity 1.34 μmol min^{-1} g, biomass 4.96 g l^{-1}) above which there is a steady decline with no or little growth at or above 45°C. However, maximum nitrilase production was attained when the organism was grown at 30°C (specific activity 1.54 μmol min^{-1} g, biomass 4.59 g l^{-1}). Specific enzyme activity and conversion of mandelonitrile also declined sharply with the increase in growth temperature above 30°C. The optimum age and volume of inoculum were found to be 12 h and 2% (v/v), respectively, for the nitrilase production by this organism (data not shown).

Effect of carbon source

Various carbon sources (final concentration 10 g l^{-1}) were tested for their effect on biomass production and specific enzyme activity. Lower enzyme activity obtained with

glucose, fructose and glycerol indicated very little or no nitrilase production probably because of catabolite repression, although they supported excellent biomass. Inorganic carbon sources such as ammonium acetate, Na-succinate or Na-citrate did not support nitrilase production in *P. putida* MTCC 5110. Nitrilase activity was found to be much higher when other carbon sources like sorbitol, mannitol, lactose and sucrose were used in the media. Starch proved to be the most suitable carbon source as it supported higher biomass as well as enhanced the specific enzyme activity when compared to the medium devoid of any carbon source (Table 1). Increasing the starch concentration beyond 5 g l^{-1} in the basal medium found to be detrimental for both the growth as well as nitrilase production by *P. putida* MTCC 5110. This could be possibly attributed to the increased viscosity in the medium because of higher starch concentration, which probably limited efficient mass transfer.

Effect of nitrogen source

Nitrogen source is an important ingredient of the medium for a microorganism. Different nitrogen sources, both organic and inorganic, were examined for nitrilase production by *P. putida* MTCC 5110 at a final concentration of 5 g l^{-1}. In general, organic nitrogen sources being more complex favoured more cell mass production compared to inorganic nitrogen sources (Table 2). The inorganic nitrogen sources also repressed the enzyme activity compared to organic sources. Soy peptone supported higher specific enzyme activity and cell mass production and was therefore chosen for further studies. The growth of the organism increased with the increasing soy peptone concentration up to 10 g l^{-1}, but the nitrilase activity decreased sharply beyond concentration level of 5 g l^{-1}.

Table 1 Effect of carbon source on growth and nitrilase production by *Pseudomonas putida*

Carbon source	Specific activity (μmol min^{-1} g)	Conversion (%)	Biomass (g l^{-1})
None	1.54±0.018	29.53±0.65	4.59±0.037
Glucose	0.12±0.021	22.20±0.73	6.98±0.042
Fructose	0.14±0.016	22.45±0.82	7.41±0.039
Sucrose	1.42±0.018	30.85±0.78	4.21±0.045
Lactose	1.58±0.022	31.35±0.69	4.55±0.039
Glycerol	0.10±0.017	21.73±0.52	7.55±0.052
Starch	1.72±0.017	35.46±0.55	5.36±0.047
Sorbitol	1.32±0.019	31.03±0.98	4.17±0.033
Mannitol	1.24±0.022	30.35±0.95	3.96±0.042
Succinate	0.28±0.01	24.83±0.89	5.23±0.038
Citrate	0.24±0.016	23.88±0.76	4.79±0.037
Acetate	ND	ND	ND

All the carbon sources were used at a concentration of 10 g l^{-1} in the basal medium described earlier. *P. putida* MTCC 5110 cells were grown for 12 h at 30°C. After harvesting the cells, the reaction with mandelonitrile was performed. Cells were harvested at regular intervals, but data at only 12 h are shown because the cells attained highest specific activity at that time only, even though medium components are changed. Enzyme activity and degree of conversion were determined by RP-HPLC. All the values are average of three readings, and the values after ± represent standard deviation
ND Not detectable

Table 2 Effect of nitrogen sources on nitrilase production by *P. putida*

Nitrogen source	Specific activity (μmol min^{-1} g)	Conversion (%)	Biomass (g l^{-1})
None	1.34±0.044	31.24±0.53	4.98±0.043
Peptone	1.78±0.019	35.44±0.85	5.90±0.057
Tryptone	0.36±0.036	23.08±0.98	6.82±0.063
Ammonium chloride	0.18±0.047	21.52±0.23	2.38±0.042
Ammonium sulphate	0.14±0.024	21.24±0.08	2.41±0.039
Ammonium nitrate	0.16±0.016	21.37±0.78	2.54±0.028
Urea	0.24±0.036	21.98±0.69	0.56±0.015

All the nitrogen sources were used at a final concentration of 5 g l^{-1} to the basal medium containing additionally 5 g l^{-1} starch. All the values are average of three readings, and the values after ± represent standard deviation

Effect of growth factors

To enhance the cell mass production, different growth factors like beef extract, yeast extract, brain heart infusion, malt extract, meat extract and yeast autolysate (final concentration 3 g l^{-1}) were examined. It was observed that all the growth factors supported abundant biomass. Both yeast extract and malt extract exhibited comparable specific enzyme activities, and when used in combination (1.5 g l^{-1} each), they exhibited higher enzyme activity and also supported heavy biomass (specific activity 2.20 μmol min^{-1} g, biomass 6.12 g l^{-1}). Therefore, a combination of yeast extract and malt extract (1.5 g l^{-1} each) as growth factor was selected for further experiments.

Effect of metal ions

The effect of various metal ions [NaCl, $CaCl_2$, $2H_2O$; Co $(NO_3)_2$, $6H_2O$; $CuSO_4$, $5H_2O$; $FeSO_4$, $7H_2O$; $MgSO_4$, $7H_2O$; $MnSO_4$, H_2O; Na_2MoO_4, $2H_2O$; $NiSO_4$, $7H_2O$; $ZnSO_4$, $7H_2O$] on the nitrilase formation was investigated. All the inorganic compounds were added separately to the medium to a final concentration of 1 mM. The addition of $FeSO_4$ caused a marked decrease in the biomass production as well as nitrilase production by the organism (specific activity 0.64 μmol min^{-1} g, biomass 2.09 g l^{-1}). Other inorganic compounds supported neither biomass nor the enzyme production, except NaCl which supported higher biomass compared to the medium devoid of any metal ions. Finally, NaCl (5 g l^{-1}) was included in the optimized

medium. In the literature, there are reports where addition of metal ions reduced the nitrilase activity as well as growth of the organism such as $CuSO_4$ which caused strong inhibition of cell growth, while $CoCl_2$ resulted in marked decrease in nitrilase level in *Rhodococcus rhodochrous* J1 (Nagasawa et al. 1988).

Effect of inducers

Nitrilases are generally known to be inducible in nature. *P. putida* MTCC 5110 cells grown in the absence of any inducer did not exhibit detectable nitrilase activity, indicating the inducible nature of the enzyme. Different compounds (nitriles, acids, amides, etc.) were tested for their ability to induce the enzyme synthesis (Table 3). Short-chain aliphatic nitriles acted as the suitable inducers for the nitrilase production in *P. putida* MTCC 5110. In some cases, these enzymes (nitrile hydrolases) are induced by the products like acids or amides (Wu and Li 2002). In the case of *P. putida* MTCC 5110, the aromatic and arylacetonitriles tested lowered both the cell mass production and activity, whereas different amides, acids and ε-caprolactam support abundant growth, but the enzyme activity was considerably lower. It was also observed that with the increase in length of the aliphatic chain, the efficiency of the induction decreased. The organism failed to grow when acrylonitrile and mandelonitrile were used as inducers. High-toxic nature of these nitriles probably hindered the growth of the microorganism. ACN was found to induce the enzyme synthesis most efficiently. The possibility of nitrilase production being induced by acetic acid, a metabolized product of ACN, was also verified, and it was observed that acetic acid did not induce nitrilase production in *P. putida* MTCC 5110. This confirmed that ACN itself and not its any metabolized product acted as inducer for the nitrilase production by this organism. ACN at a concentration of more than 3 g l^{-1} was detrimental to the growth of the organism (specific activity 1.89 μmol min^{-1} g, biomass 3.09 g l^{-1}). We selected 0.5 g l^{-1} ACN as optimum inducer concentration for further optimization studies (data not shown).

Table 3 Effect of inducers on growth and nitrilase production by *P. putida*

Inducer	Specific activity (μmol min^{-1} g)	Conversion (%)	Biomass (g l^{-1})
None	0.04±0.014	ND	6.49±0.059
Acetonitrile	2.50±.027	43.83±0.77	7.73±0.054
Propionitrile	2.34±0.035	40.56±0.89	6.75±0.063
Butyronitrile	1.80±0.021	39.04±0.74	7.28±0.067
Valeronitrile	2.04±0.031	40.82±0.67	5.50±0.058
Isobutyronitrile	2.20±0.024	38.41±0.78	6.12±0.071
Isovaleronitrile	1.48±0.015	32.47±0.89	6.33±0.074
Phenyl acetonitrile	1.78±0.020	40.92±0.84	1.92±0.048
2-Thiophene acetonitrile	0.88±0.09	27.42±0.98	2.12±0.051
Acetamide	1.46±0.017	32.28±0.73	7.39±0.067
Benzamide	1.44±0.021	32.14±0.79	6.68±0.061
Mandelamide	1.88±0.023	35.75±0.64	6.39±0.075
Acetic acid	ND	ND	4.64±0.041
Benzoic acid	1.42±0.018	31.63±0.78	6.88±0.039
Mandelic acid	1.26±0.015	30.67±0.42	6.64±0.076
Phenylacetic acid	1.22±0.017	30.07±0.37	6.43±0.064
ε-Caprolactam	1.50±0.017	32.53±0.59	8.24±0.081

All the inducers were used at a final concentration of 1 g l^{-1}. All the values are average of three readings, and the values after ± represent standard deviation
ND Not detectable

Effect of inducer feeding

The volatile nature of ACN and its slow metabolism into acetic acid resulted in its gradual elimination from the culture medium during cultivation which might lower induction efficiency. Under these circumstances, effect of ACN addition during the course of cultivation had high impact on nitrilase production. Since, *P. putida* MTCC 5110 cells exhibited highest nitrilase activity after 12 h of cultivation, ACN addition was delayed by 6 and 9 h in the culture broth during cultivation with final concentration constant at 0.5 g l^{-1} (Fig. 2a). Around 1.35-fold enhancement in specific activity and 1.11-fold increase in degree of conversion of mandelonitrile were observed when ACN addition was delayed by 6 h compared to control (where ACN was added at 0 h; Fig. 2c). Furthermore, we investigated the repeated feeding of ACN during the course of cultivation. Three modes of feeding were adopted: (a) feeding at 3, 6 and 9 h with final concentration constant at 0.5 g l^{-1}; (b) feeding of 0.5 g l^{-1} each time (3, 6 and 9 h); and (c) feeding of 0.5, 1.0 and 2 g l^{-1} at 3, 6 and 9 h, respectively. The activity of nitrilase because of this continuous ACN feeding increased slightly when compared to addition of inducer at 0 h, but the enhancement was not significant ($P<0.05$; Fig. 2b). Thus, addition of ACN at 6 h was found to be optimum for nitrilase production by *P. putida* MTCC 5110.

SDS-PAGE analysis

The enhancement of nitrilase formation was also demonstrated by SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the CFEs obtained after lysis of the cells grown under different culture conditions (Fig. 3a). The nitrilase was detected in the gel by comparison with the purified nitrilase as a marker. A very faint band of nitrilase was observed in lane 3 corresponding to the CFE when cells were grown in the absence of any inducer, implying its very little constitutive expression. Analysis of variance (ANOVA) revealed that the differences in the mean values of the nitrilase band intensities between feeding at 0 and 6 h were greater than would be expected by chance, and that there was a statistically significant difference ($P<0.01$). As revealed by the quantitative analysis of the gel tracks by densitometric

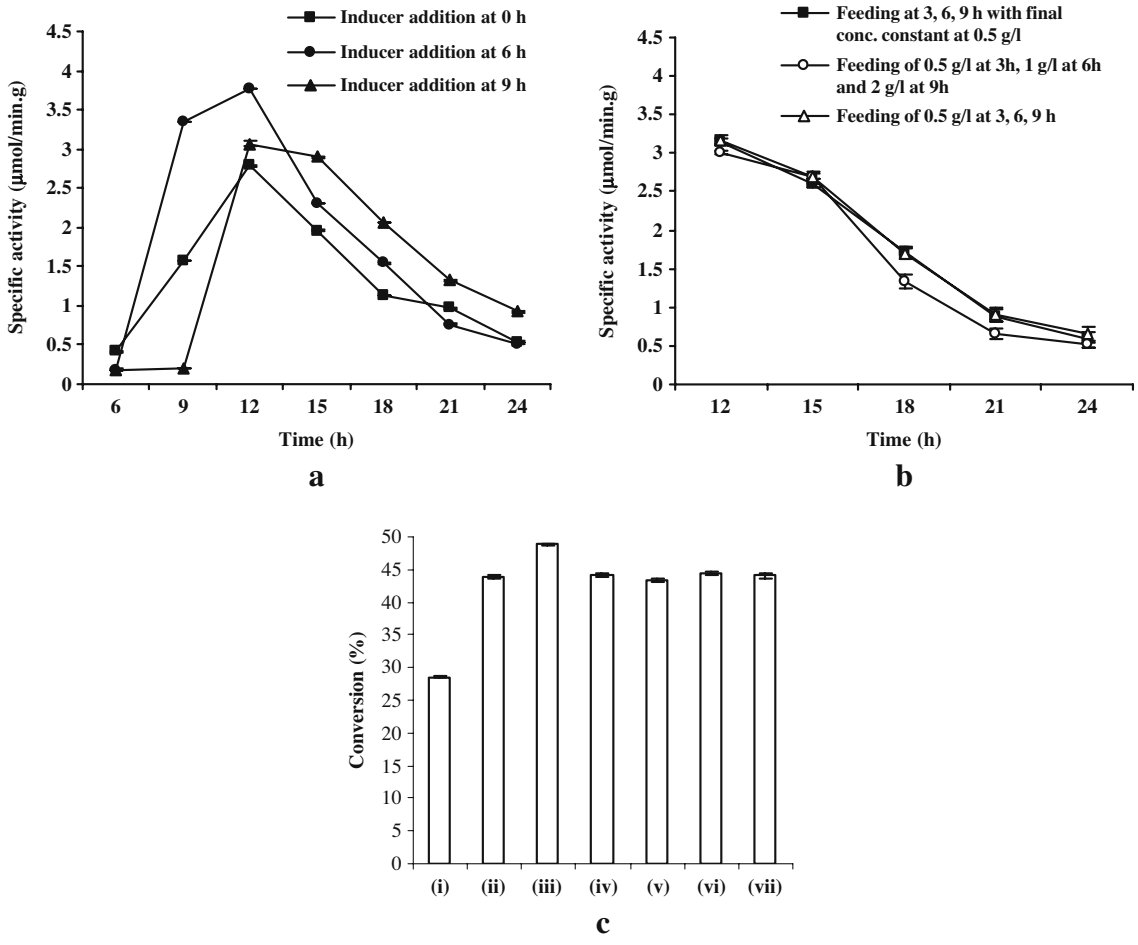

Fig. 2 Effect of induction time on nitrilase production. **a** Specific activity profile when acetonitrile (ACN) was added only once but at different time. **b** Specific activity profile with continuous feeding of ACN. **c** Degree of mandelonitrile conversion with and without ACN addition: (*i*) before optimization, (*ii*) ACN addition at 0 h in optimized medium, (*iii*) ACN addition at 6 h, (*iv*) ACN addition at 9 h, (*v*) continuous feeding of ACN at 3, 6 and 9 h with final concentration constant at 0.5 g l^{-1}, (*vi*) continuous feeding of 0.5 g l^{-1} at 3, 6 and 9 h, and (*vii*) continuous feeding of 0.5 g l^{-1} at 3 h, 1 g l^{-1} at 6 h and 2 g l^{-1} at 9 h

analysis, the intensities of the nitrilase band in other lanes are in the order of lane 4>lane 7>lane 6>lane 5 (Fig. 3b). It appeared that band intensity of the nitrilase in lane 4 is 1.84- and 1.58-fold higher than that in lanes 6 and 7, respectively, essentially revealing higher production of the enzyme when ACN addition was delayed by 6 h.

Enantioselective hydrolysis of mandelonitrile

After achieving significant enhancement in nitrilase titre with optimization of cultivation conditions, *P. putida* MTCC 5110 cells were grown under optimal conditions and were further used for enantioselective hydrolysis of mandelonitrile in both free and immobilized forms. Immobilization on the surfaces of porous materials (celite, silica, etc.) generally produced catalyst with low specific activity, and rapid loss of activity was observed due to attrition of the particle surface during mixing (Monsan et al. 1987). Entrapment seemed to be a better choice for immobilization, though employed matrices other than alginate rapidly deactivate the enzyme because of high temperature required for gelation. Also, alginate beads are not as much shear sensitive as other matrices, and therefore, much higher production rates of catalyst beads were achieved (Monsan et al. 1987). Considering all this, *P. putida* MTCC 5110 cells were immobilized in Ca-alginate beads and used for further nitrile hydrolysis.

Effect of pH and temperature on nitrile hydrolysis

Considering the spontaneous decomposition and in situ racemization of unreacted mandelonitrile via benzaldehyde and hydrogen cyanide formation (Fig. 1) at slightly alkaline pH (Inagaki et al. 1992), it was assumed that pH of the reaction mixture would have profound effect on overall nitrile hydrolysis. This was evident from the fact that the conversion of mandelonitrile increased from 63.87 and 56.45% at pH 7.0 to 95.35 and 93.2% at pH 7.5 in the case of free and immobilized cells, respectively. This could be attributed to better decomposition and racemization rates of

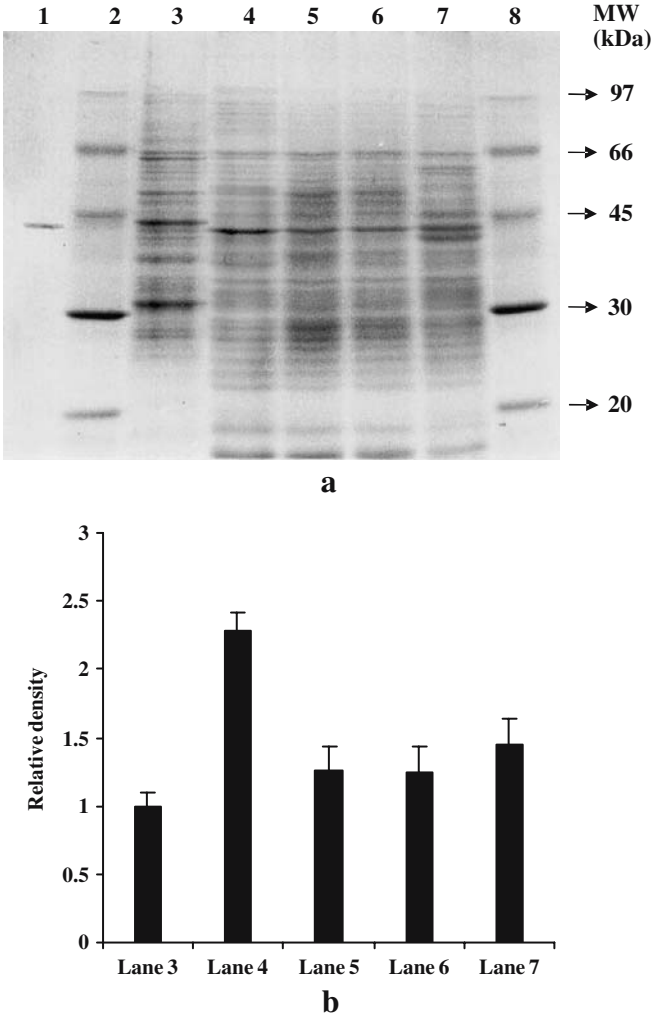

Fig. 3 **a** Protein profiles of cell-free extract (CFE) on 12% sodium dodecyl phosphate-polyacrylamide gel electrophoresis (SDS-PAGE). *Lane 1*: purified nitrilase from *P. putida*, *lanes 2 and 8*: molecular weight markers (Phosphorylase b, 97 kDa; Albumin, 66 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 30 kDa; Trypsin inhibitor, 20 kDa), *lane 3*: CFE when cells were grown in the basal medium without inducer before optimization, *lane 4*: CFE when cells were grown in the optimized medium with ACN added at 6 h, *lane 5*: CFE when cells were grown in the basal medium with isobutyronitrile as inducer, *lane 6*: CFE when ACN was added at 0 h in the optimized medium, *lane 7*: CFE when ACN was continuously fed at 3, 6 and 9 h with final concentration constant at 0.5 g l^{-1}. All the lanes were loaded with equal amount of protein (30 μg). **b** The relative nitrilase band intensities observed employing different inducer feeding strategies. The band intensities are expressed relative to the control (with no inducer; *lane 3*) taken as 1

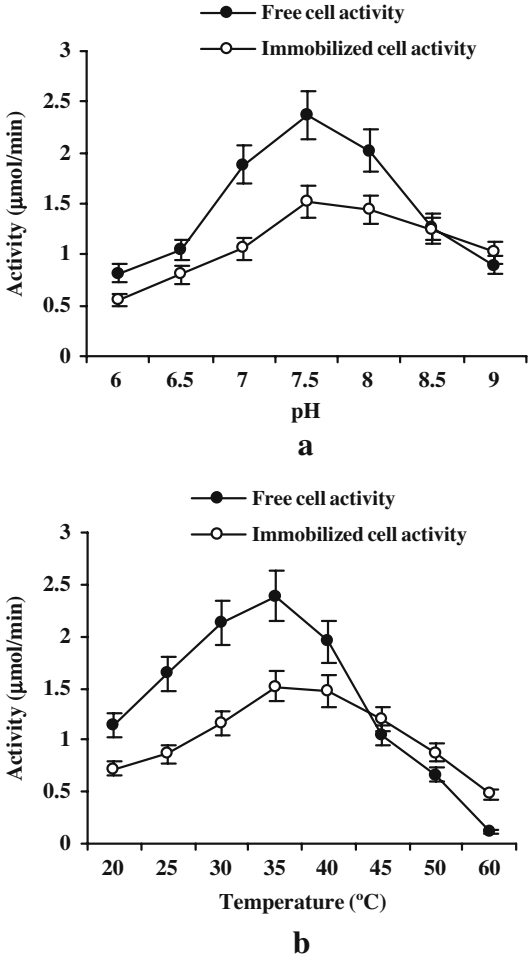

Fig. 4 Effect of pH (**a**) and temperature (**b**) on initial rate of nitrile hydrolysis by free and immobilized cells of *P. putida*

the unreacted mandelonitrile which were favored by alkaline conditions. Initial reaction rates (activity) measured for immobilized cells were approximately 1.56- to 1.77-fold lower than those obtained with free cells at these pH values (Fig. 4a). This was assumed to be due to the mass transfer limitations imposed by the immobilization matrix. However, immobilization provides cells an additional degree of thermal stability which was evident from the higher activity values at temperatures more than 45°C (Fig. 4b).

Kinetic model in the immobilized cell systems

For cells entrapped in a microporous matrix such as calcium alginate, the substrate diffuses through the gel to react with the enveloped cells. Thus, diffusion and catalysis take place simultaneously in this situation. However, the rate of reaction depends strongly on the diffusitivities of substrate within the matrix. Assuming that the cells are uniformly distributed in a spherical bead and there is no substrate partition between the exterior and interior of the bead, the following equation may be applied according to the Fick's law at steady state:

$$\frac{3600}{1000}\frac{D_e}{\rho_P}\left[\frac{d^2C}{dr^2}+\frac{2}{r}\frac{dC}{dr}\right]=v \qquad (1)$$

where C is the nitrile concentration within the bead (mg l^{-1}), r is the radial position within the bead (cm), D_e is the effective diffusivity of nitrile within the bead (cm^2 s^{-1}) and ρ_p is the density of dried cells (g cm^{-3}). Assuming that the reaction obeys first-order kinetics (which is correct at least at lower substrate concentrations), the relationship between v

and C is:

$$v = kC \tag{2}$$

where k is the first-order reaction rate constant ($cm^3\ g^{-1}\ h$).

Solving Eq. (1) with boundary conditions $C=C_S$ at $r=R$ and $dC/dr=0$ at $r=0$, we obtain:

$$\frac{C}{C_s} = \frac{\sin h(\phi r/R)}{(r/R)\sin h\phi} \tag{3}$$

where R is the radius of the bead (cm), C_s is the substrate concentration at the bead surface and ϕ is the dimensionless group (Thiele modulus for a spherical particle) defined by Eq. (4).

$$\phi = R\sqrt{\frac{k'}{D_e}} \tag{4}$$

where k' is the rate constant ($k'=k\times\rho_P$).

In the presence of diffusion limitation, the rate of reaction is expressed in terms of effectiveness factor (η) defined as the ratio of actual reaction rate to that without diffusion limitation (or the rate evaluated at the outer surface, v_s), or

$$v = \eta v_s \tag{5}$$

The value of η is a measure of mass transfer effect. For $\eta<1$, the process is diffusion limited. Assuming that actual reaction rate (v) equals the diffusion rate of substrate into the bead at $r=R$, we obtain:

$$n = \frac{3}{\phi}\left[\frac{1}{\tan h\phi} - \frac{1}{\phi}\right] \tag{6}$$

The relative importance of diffusion and reaction rate is highlighted in Eq. (6). η approaches 1 for small values of ϕ, and the intraparticle diffusion has no effect on the enzymatic reaction. That is, the enzyme catalyzed reaction predominantly controls the rate. On the other hand, the intraparticle diffusion has large effect on the reaction for $\phi>5$ (Tanaka et al. 1984; Chen et al. 1993).

Influence of cell loading and bead diameter on the efficiency of immobilization system

Amount of biomass loading in the reaction mixture also affects the degree of bioconversion as well as the reaction rate. Effectiveness (η) of the immobilization system which was defined as the enzyme activity of immobilized cells with respect to enzyme activity of free cells (Jamuna and Ramakrishna 1992), determined under the same conditions, reflected that 1.66 g of cells being loaded per gram of alginate was the optimal system for nitrile hydrolysis by *P. putida* MTCC 5110 (Fig. 5). Further increase in the cell loading lowered the effectiveness possibly because of diffusion restriction of the substrate. Enhancement in the reaction velocities of the immobilized system could be achieved by employing smaller bead sizes. At lower bead diameters, the effectiveness of the immobilized system was found to approach that of the free cell ($\eta=1$). The greater effective surface area provided by beads of lower bead diameter was found to aid the mass transfer of the substrate into the beads harbouring the biocatalyst (Table 4). Since η was not very much smaller than 1 (0.65 and 0.56 for 0.16- and 0.2-cm bead diameter, respectively), it may be concluded that the reaction is diffusion limited to some extent only. However, with increase in bead size (>0.2 cm), there appeared to be greater mass transfer problems ($\phi>5$), which was also reflected by the values of diffusivities. For hydrophobic substrates such as mandelonitrile which is sparingly soluble in aqueous medium, obtaining low diffusion coefficients is hardly surprising.

Recycling

To investigate the effect of recycling on the degree of mandelonitrile conversion, biotransformation reaction was carried out both with free and alginate-entrapped cells in batch mode. Each time, the hydrolytic reaction was carried out for 4 h at 35°C. With the increase in number of cycles, the conversion ratio became lower for free cells, and around 60% conversion was achieved after 5 cycles, with little or no conversion at or above nine batches. On the contrary, immobilized preparation retained approximately 94% of the initial activity even after 20 consecutive batches (Fig. 6). Further improvement in the reusability of the immobilized biocatalyst can be achieved by cross-linking the alginate beads with suitable agents.

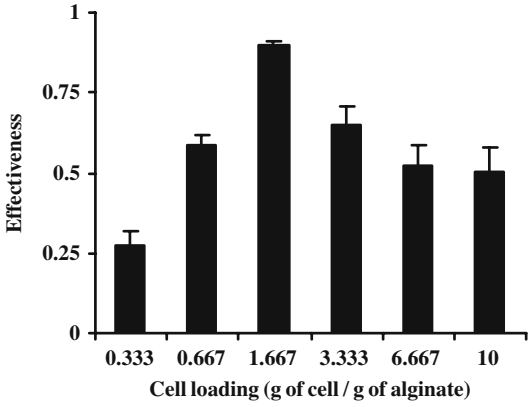

Fig. 5 Effectiveness of the immobilized cell system at different cell loading

Table 4 Analysis of the immobilization system based on different bead diameters

d_P (cm)	ν (mg g^{-1} h^{-1})	η	ϕ	k'×10^{-3} (cm^3 cm^{-3} s^{-1})	D_e×10^{-6} (cm^2 s^{-1})
Free cell	43.54±1.57	1	–	–	–
0.16	28.36±0.89	0.65	3.1	2.65	1.76
0.2	24.42±0.82	0.56	4	2.28	1.42
0.26	20.04±0.73	0.46	5.3	1.87	1.12
0.32	17.04±0.85	0.39	6.4	1.59	0.99

Preparative scale biotransformation

Finally, a preparative scale biotransformation reaction in fed batch mode was set up for the production of enantiopure (*R*)-(-)-mandelic acid employing alginate-entrapped *P. putida* MTCC 5110 cells. The reaction mixture (100 ml) consisted of 0.2 g mandelonitrile and optimized amount of biocatalyst to alginate ratio in 0.1 M Tris–HCl buffer (pH 7.5). After every 4 h, 0.2 g of the nitrile was fed into the system. Feeding of the substrate was carried out to relieve the biocatalyst from further substrate inhibition. Moreover, the reaction rate remained constant over the entire fed batch operation, suggesting no product inhibition of the nitrilase. Around 1.95 g (*R*)-(-)-mandelic acid was recovered from the reaction mixture after 40 h (Fig. 7). The mandelic acid formed was found to be 98.8% enantiomerically pure as determined by polarimeter. The volumetric productivity (g mandelic acid l^{-1} h^{-1}) and the catalyst productivity (g mandelic acid g^{-1} cell) were found to be 0.49 and 0.39, respectively.

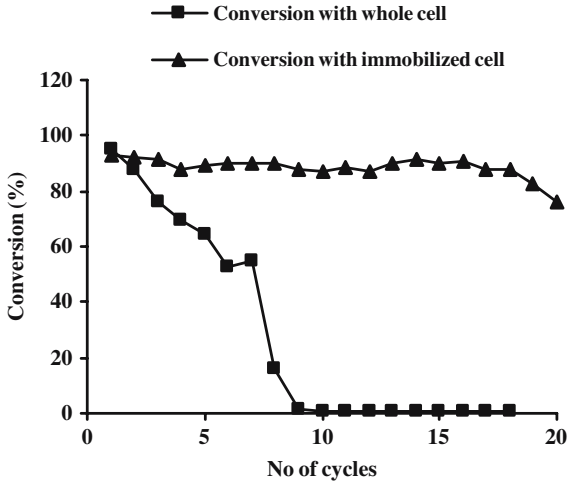

Fig. 6 Reusability of the free and immobilized cell system

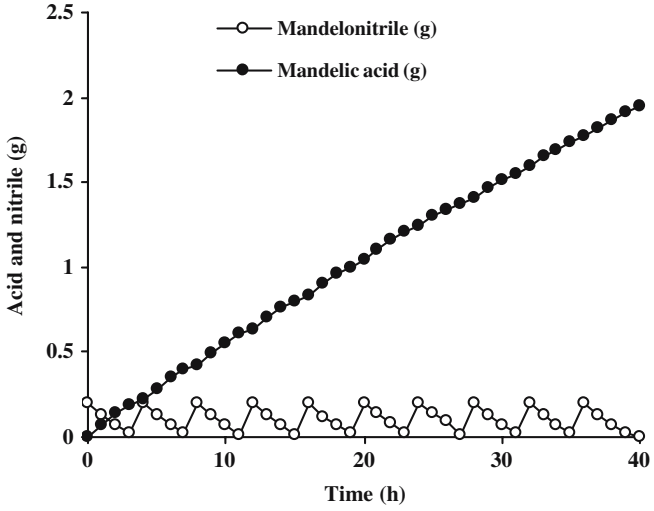

Fig. 7 Time course of the fed batch reaction for the production of (*R*)-(−)-mandelic acid by immobilized cells of *P. putida*

Discussion

Based on the above results, a medium containing 5 g each of starch, soy peptone and NaCl, 1.5 g each of yeast extract and malt extract per liter of water with ACN (0.5 g l^{-1}) added after 6 h of inoculation seemed to be most suitable for high cell growth and nitrilase activity. The enzyme system (nitrilase) responsible for the hydrolysis of mandelonitrile by *P. putida* MTCC 5110 is inducible in nature. The nitrilase is not active on aliphatic nitriles (unpublished work); however, the short-chain aliphatic nitriles acted as efficient inducers. They also acted as efficient inducers for nitrilase expression in other micro-organisms; for example, isovaleronitrile induced nitrilase in *Alcaligenes faecalis* JM3 (Mauger et al. 1990) and *R. rhodochrous* K22 (Kobayashi et al. 1991), butyronitrile acted as inducer for arylacetonitrilase in *A. faecalis* ATCC 8750 (Yamamoto et al. 1991), ACN efficiently induced nitrilase in *Fusarium oxysporum* (Goldhust and Bohak 1989) and ε-caprolactam caused hyper-induction of nitrilase in *R. rhodochrous* J1 (Nagasawa et al. 1990). It was proposed that short-chain aliphatic nitriles bind more efficiently to the same site of repressor protein where substrate binding takes place (Nagasawa et al. 1987). It was also observed that in the case of *P. putida* MTCC 5110 with increase in chain length, the efficiency of induction decreases. In some cases, these enzyme systems (nitrile hydrolases) were also induced by the products like acids or amides (Wu and Li 2002). But in the case of *P. putida*, the acids and amides tested did not act as efficient inducers. ACN was found to induce the enzyme synthesis most efficiently. The activity of the purified nitrilase towards ACN is only 2% with respect to mandelonitrile (unpub-

lished results). This slow metabolism of ACN in the culture broth possibly accounted for the high induction of nitrilase by ACN. High concentration of ACN could not be used in the culture medium since at higher concentration, it became detrimental to cell growth. Due to its volatile nature and slow metabolism to acetic acid, ACN is gradually eliminated from the culture medium during cultivation. It was observed that addition of ACN after 6 h of inoculation increased the enzyme activity by approximately 1.35-fold compared to when ACN was added at the start. Similar enhancement in nitrilase production was achieved in the case of *Rhodococcus* sp. CGMCC 0497 when addition of methacrylamide was delayed by 24 h (Wu and Li 2002) and in the case of *R. rhodochrous* J1 (Nagasawa et al. 1988) and *R. rhodochrous* K22 (Kobayashi et al. 1991) where isovaleronitrile was repeatedly fed in the medium during cultivation. Further studies need to be carried out to elucidate the mechanism of induction in these systems. Most inducible enzymes are generally subjected to catabolite repression when simple sugars are used as carbon sources. The nitrilase of *P. putida* MTCC 5110 was strongly repressed by simple sugars such as glucose, fructose, etc. Starch appeared to be the most effective carbon source. Inorganic sources such as ammonium acetate, Na-succinate or Na-citrate did not support nitrilase production, although these acted as preferable carbon sources for *A. faecalis* ATCC 8750 (Yamamoto et al. 1991) and *Acinetobacter* sp. AK 226 (Yamamoto and Komatsu 1991). Soy peptone and a combination of yeast extract and malt extract acted as better nitrogen source and growth factors, respectively. The growth of the organism, nitrilase activity and the conversion of mandelonitrile in the reaction mixture were increased by 1.5-, 36.9- and 1.71-fold, respectively, using different medium engineering techniques. The optical purity of mandelic acid formed was determined at different stages of medium optimization by analysing the product on a Chiralcel OD-H column. Unlike Wu and Li (2002), we did not observe any significant change in the ee (%) values of the (*R*)-(-)-mandelic acid formed due to variation in culture conditions.

Since most of the cost associated with a biocatalytic process is related to the biocatalyst generation, increased frequency of catalyst replacement due to its unstable nature is reflected by high cost associated with such processes. Hence, increasing the stability and therefore the reusability of the biocatalyst is of high significance which can be further achieved by immobilization of the catalyst on a suitable support. *P. putida* MTCC 5110 cells harbouring nitrilase was therefore further immobilized in calcium alginate beads, and the immobilization efficiency was verified by comparing the rate of nitrile hydrolysis with that of free cells. Effect of pH suggested that the substrate recycling via spontaneous degradation was more effective at alkaline pH (>8.0); however, enzyme functioned best at pH 7.5. The rate of nitrile hydrolysis was much lower in the case of the immobilized system, though it offered higher degree of thermal stability to the cells. Analysis of the mass transfer through immobilized system indicated a greater diffusional resistance at high biocatalyst loading and bead diameters. At lower bead diameters, the effectiveness of the immobilized system was found to approach that of the free cell (η=1). The greater effective surface area provided by beads of lower bead diameter was found to aid the mass transfer of the substrate into the beads harbouring the biocatalyst. Additionally, the system was also characterized based on the Thiele modulus (ϕ). The results presented allowed estimation of effective diffusion coefficients, which may be of help for designing a suitable reactor to lift the existing mass transfer limitations. Efficient biocatalyst recycling was achieved as a result of immobilization with immobilized cells exhibiting 88% conversion even after 20 batch recycles. After a thorough characterization, the alginate-entrapped cells were reacted with mandelonitrile with intermittent feeding of the substrate to produce 1.95 g of (*R*)-(-)-mandelic acid with an ee of 98.8%. The results suggest that the biocatalyst described here fulfills the requirement for large-scale low-cost production of this important fine chemical due to its greater stability, efficient reusability and precise stereo control.

The successful integration of this unwavering nitrilase into a sizeable production plant would require a scaleable production technology. Utilizing the optimized medium and adopting a strategy such as inducer feeding approach, large volumes of biomass can be generated in stirred tank reactor with enhanced nitrilase activity (work under progress). We are also working on the development of an immobilized cell reactor for the large-scale production of (*R*)-(-)-mandelic acid. Further mechanistic and genetic level studies are required to elucidate the substrate specificity as well as mechanism of induction of this enzyme.

Acknowledgement A. Banerjee and P. Kaul gratefully acknowledge the fellowship provided by CSIR, Government of India. This is NIPER communication number 346.

References

Aksu Z, Bulbul G (1999) Determination of effective diffusion coefficient of phenol in Ca-alginate immobilized *P. putida* beads. Enzyme Microb Technol 25:344–348

Banerjee A, Sharma R, Banerjee UC (2002) Nitrile degrading enzymes: current status and future prospects. Appl Microbiol Biotechnol 60:33–44

Banerjee A, Kaul P, Sharma R, Banerjee UC (2003) A high-throughput amenable colorimetric assay for enantioselective screening of nitrilase producing microorganisms. J Biomol Screen 8:559–565

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Brenner C (2002) Catalysis in the nitrilase superfamily. Curr Opin Chem Biol 12:775–782

Chen D, Lewandowski Z, Roe F, Surapaneni P (1993) Diffusivity of Cu^{2+} in Ca-alginate gel beads. Biotechnol Bioeng 41:755–760

Goldhust A, Bohak Z (1989) Induction, purification and characterization of the nitrilase of *Fusarium oxysporum*. Biotechnol Appl Biochem 11:581–601

Inagaki M, Hiratake J, Nishioka T, Oda J (1992) One pot synthesis of optically active cyanohydrin acetates from aldehydes via lipase catalyzed kinetic resolution coupled with in situ formation and racemization of cyanohydrins. J Org Chem 57:5643–5649

Jamuna R, Ramakrishna SV (1992) Continuous synthesis of thermostable α-amylase by *Bacillus* cells immobilized in Ca-alginate. Enzyme Microb Technol 14:36–41

Kakeya H, Sakai N, Sugai T, Ohta H (1991) Preparation of optically active α-hydroxy acid derivatives by microbial hydrolysis of cyanohydrins and its application to the synthesis of (*R*)-4-dodecanolide. Agric Biol Chem 55:1877–1881

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

Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1991) Hyperinduction of an aliphatic nitrilase by *Rhodococcus rhodochrous* K22. FEMS Microbiol Lett 77:121–124

Lammeli UK (1970) Cleavage of structural proteins during the assembly of the head the bacteriophage T4. Nature 227:680–685

Layh N, Stolz A, Forster S, Effenberger F, Knackmuss H-J (1992) Enantioselective hydrolysis of *O*-acetylmandelonitrile to *O*-acetylmandelic acid by bacterial nitrilases. Arch Microbiol 158:405–411

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

Monsan P, Durand G, Navarro JM (1987) Immobilization of microbial cells by adsorption to solid supports. Methods Enzymol 135:307–318

Mylerova V, Martinkova L (2003) Synthetic applications of nitrile converting enzymes. Curr Org Chem 7:1–17

Nagasawa T, Nanba H, Ryuno K, Takeuchi K, Yamada H (1987) Nitrile hydratase of *Pseudomonas chlororaphis* B23: purification and characterization. Eur J Biochem 162:691–698

Nagasawa T, Kobayashi M, Yamada H (1988) Optimum culture conditions for the production of benzonitrilase by *Rhodococcus rhodochrous* J1. Arch Microbiol 150:89–94

Nagasawa T, Nakamura T, Yamada H (1990) ε-Caprolactam, a new powerful inducer for the formation of *Rhodococcus rhodochrous* J1 nitrilase. Arch Microbiol 155:13–17

O'Reilly C, Turner PD (2003) The nitrile family of CN hydrolysing enzymes—a comparative study. J Appl Microbiol 95:1161–1174

Sugai T, Yamazaki T, Yokoyama M, Ohta H (1997) Biocatalysis in organic synthesis: the use of nitrile and amide hydrolysing microorganisms. Biosci Biotechnol Biochem 61:1419–1427

Tanaka H, Matsumura M, Veliky IA (1984) Diffusion characteristics of substrates in Ca-alginate gel beads. Biotechnol Bioeng 26:53–58

Watanabe I, Satoh Y, Enomoto K, Seki S, Sakshita K (1987) Optimal conditions for cultivation of *Rhodococcus* sp. N774 and conversion of acrylamide by resting cells. Agric Biol Chem 51:3201–3206

Wu ZL, Li ZY (2002) Enhancement of enzyme activity and enantioselectivity via cultivation in nitrile metabolism by *Rhodococcus* sp. CGMCC 0497. Biotechnol Appl Biochem 35:61–67

Yamada H, Ryuno K, Nagasawa T, Enomoto K, Watanabe I (1986) Optimum culture conditions for production of nitrile hydratase by *Pseudomonas chlororaphis* B23. Agric Biol Chem 50:2859–2865

Yamamoto K, Komatsu K (1991) Purification and characterization of nitrilase responsible for the hydrolysis from *Acinetobacter* sp. AK 226. Agric Biol Chem 55:1459–1466

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