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Screening for enantioselective nitrilases: kinetic resolution of racemic mandelonitrile to (*R*)-(−)-mandelic acid by new bacterial isolates

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Abstract—Several new microorganisms have been isolated with high nitrilase activity against (*RS*)-mandelonitrile using the enrichment culture technique. The organisms were cultivated in liquid culture and the enzyme activity was determined at different phases of growth. The organisms having high enzyme titre were further grown and used as catalysts for the transformation of mandelonitrile to mandelic acid. The percentage conversion was checked with RP-HPLC and the enantiomeric excess was determined on a chiral column. Three isolates gave the desired product, (*R*)-(−)-mandelic acid with high ee (%) and were identified as *Pseudomonas putida*, *Microbacterium paraoxydans* and *Microbacterium liquefaciens*. All three isolates showed good specific activity (0.33–0.50 U/mg min) with high ee (>93%) and *E* values. The conversion of racemic mandelonitrile to mandelic acid by these isolates was compared: *P. putida* was found to be the most suitable biocatalyst for further studies as it showed higher reaction rate (k_{Rxn}), lower K_m, better growth rate (μ), good yield and ee values and higher stability compared to the other two microorganisms.

1. Introduction

Hydrolase catalyzed reactions have been widely applied in organic synthesis. Nitrilases (EC 3.5.5.1) are an important class of hydrolases that convert nitriles to the corresponding carboxylic acids and ammonia. Nitriles may also follow a bienzymatic pathway for the conversion to acids, involving nitrile hydratases (EC 4.2.1.84) and amidases (EC 3.5.14). Nitrile hydratases (NHase) catalyze the hydration of nitriles to the corresponding amides, followed by their conversion to acids and ammonia by amidases. Nitrilases are expected to be useful biocatalysts for organic synthesis because this ecofriendly bioconversion allows clean and mild synthesis with high selectivity and yield. Early reports generally described nitrilases as fairly specific for aromatic nitriles.[1,2] However, recent reports include nitrilases, which are active on aliphatic as well as arylacetonitriles.[3] The major disadvantage for the use of nitrilases in industrial applications is their relatively poor stability.[4] The presence of a critical thiol (–SH) group at the active site of the enzyme may contribute to this instability.[5] Greater stability of these enzymes would certainly give them better and higher utility for industrial applications. To date, many nitrilases, nitrile hydratases and amidases were reported from a variety of microorganisms.[6] The majority of these enzymes are highly enantioselective to give enantiopure product in high yield.

A major problem in the development of a specific biotransformation is to find the appropriate biocatalyst. The ideal catalyst is generally considered in terms of turn over number and specificity constant. However, each biocatalytic process is constrained by a set of parameters (properties of substrate, product and bioconversion reaction, etc.) and the catalyst must fit into this category to give the process designer more flexibility.[7] If there are no commercially available enzyme preparations, the desired activities can be found either by screening of strains from culture collections or by isolation of new microorganisms via enrichment techniques. In the present investigation, our main aim is to present new bacterial strains, which enantioselectively hydrolyze racemic mandelonitrile to (*R*)-(−)-mandelic acid. (*R*)-(−)-Mandelic acid is a key intermediate for the

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production of semi-synthetic cephalosporins and penicillins.[8,9] It is also used as a chiral resolving agent[10] and chiral synthon for the synthesis of anti-tumour agents,[11] anti-obesity agents,[12] etc. (*R*)-(−)-Mandelic acid is presently produced by optical resolution of the racemate with chiral amines.[13] Various enzymatic routes for production of enantiomerically pure α-hydroxy carboxylic acids have been reviewed elsewhere,[14] but these production methods involving lipase,[15] esterase,[16] glyoxalase,[17] mandelate dehydrogenase[18] and mandelate racemase[19] have not been used for industrial production because of expensive substrates, involvement of co-factors, cost associated with the production of enzymes and above all because of operational stability of the biocatalyst in question. The newly found biocatalysts may be useful tools for the production of this important chiral building block.

The objective of the present study was to access enzyme systems that harbour kinetic as well as operational superiority for the resolution of racemic mandelonitrile to (*R*)-(−)-mandelic acid, giving high conversion, with high selectivity in minimum possible time and are stable under a range of conditions for a more flexible process viability study.

2. Results and discussion

2.1. Screening of microorganisms

Around 60 isolates were obtained from soil samples by the enrichment culture technique in minimal salt medium (MSM) containing phenylacetonitrile (2 mM) as the sole source of carbon and nitrogen. Mandelonitrile could not be used as substrate since at pH 7.0, it spontaneously degrades to benzaldehyde and HCN. Thus enrichment with mandelonitrile as sole source of carbon and nitrogen would result in microorganisms able to use HCN. After initial screening by the pH responsive method,[20] three new bacterial isolates, which showed desired selectivity, were selected for further studies. The microorganisms were identified as *Pseudomonas putida*, *Microbacterium paraoxydans* and *Microbacterium liquefaciens* by 16S rRNA sequence analysis, chemotaxonomical analysis and different biochemical tests. The nitrilase activities of the whole cell biocatalysts were in the range of 0.3–0.5 U/mg min as determined by the fluorescence method.[21]

While *P. putida* showed maximum activity around 12 h of growth period, both *M. paraoxydans* and *M. liquefaciens* exhibited maximum activity around 24 h. It is important to note that further optimization of cultivation conditions, such as media composition and physicochemical parameters, etc. are necessary to achieve higher enzyme activities.

Since thermostability is an index of biocatalyst stability, studies were also performed with the newly isolated microorganisms at 30 °C in order to find out the stability

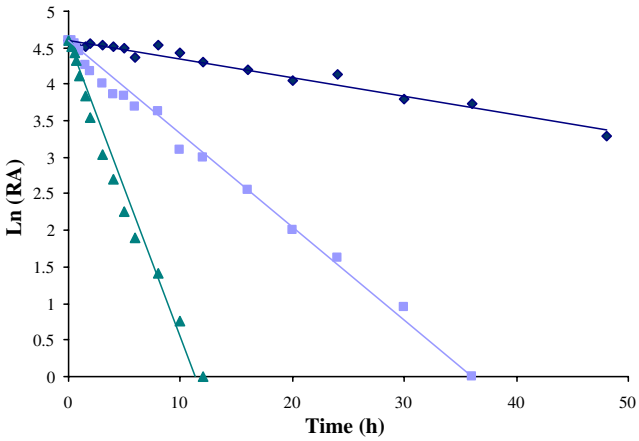

Figure 1. Thermostability profiles of the biocatalysts at 30 °C. ◆ *P. putida*; ■ *M. liquefaciens*; ▲ *M. paraoxydans*. Cells were harvested by centrifugation (5000 × g) and suspended in phosphate buffer (0.1 M, pH 7.0). The cell suspensions were incubated at 30 °C and a fixed quantity (1 mL) of cell suspension were withdrawn at different time intervals. To the cell suspension (50 mg/mL) 2 mM mandelonitrile (from a stock of 100 mM in ethanol) was added and incubated at 30 °C. The reaction was stopped after 10 min with the addition of 100 μL 1 N HCl and the amount of mandelic acid formed was determined by RP-HPLC (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 with a solvent system 0.01 M phosphate buffer (pH 4.8) and methanol (65:35, v/v). The retention times for mandelic acid and mandelonitrile were 6.3 and 22.5 min, respectively. $A_{254\,nm}$ was measured. Residual activity (RA) was calculated in accordance with the activity at 0 h. Ln(RA) was plotted against time. The slopes of the individual line correspond to K_{Deact} of individual microorganisms at the specified temperature.

of the biocatalysts under operational conditions (Fig. 1). Studies revealed that the nitrilase of *P. putida* has a much higher half-life ($t_{1/2}$ 27.28 h) than *M. paraoxydans* ($t_{1/2}$ 1.70 h) and *M. liquefaciens* ($t_{1/2}$ 5.41 h) (Table 1).

2.2. Enantioselective conversion of mandelonitrile

The resting cells of bacterial isolates were incubated with racemic mandelonitrile and product formation and enantiomeric purity were determined by RP-HPLC and chiral HPLC, respectively. In the case of all the three isolates, the ee (%) values were very high at the start of the reaction, but dropped down as the reaction proceeds and more product formation occurred (data not shown). All the three isolates showed desired enantioselectivity [(*R*)-(−)-mandelic acid] with high percentage conversion (Table 2). The rate of nitrile hydrolysis for the three isolates was visualized using a pH sensitive indicator based colorimetric assay[20] as shown in Figure 2.

In the case of *P. putida* the colour turn over (green to yellow) was much faster than the other two microorganisms indicating the faster reaction rate of the former. To confirm the observed high reaction rates the reaction course was also monitored by HPLC (Fig. 3).

Around 30% conversion was achieved only in 10 min with *P. putida* cells, whereas *M. paraoxydans* and

Table 1. Comparison of short listed isolates based on different biochemical properties

Organism[d]	Gram character[e]	Specific activity (U/mg min)	μ (h^{-1})	$t_{1/2}$[f] (h)	K_{Deact}[g] (h^{-1})
P. putida	(+)ve	0.3327[a]	0.46	27.28	0.0254
M. paraoxydans	(+)ve	0.3086[b]	0.31	1.70	0.4071
M. liquefaciens	(+)ve	0.5072[c]	0.36	5.41	0.1279

Microorganisms were isolated by enrichment culture in minimal salt medium (MSM) of following composition (g/L): Na_2HPO_4, 2; KH_2PO_4, 1; NH_4Cl, 0.4; $MgCl_2$, 0.4 and phenylacetonitrile 2 mM.

Microorganisms were cultivated in the medium of following composition (g/L): peptic digest of animal tissue, 5; NaCl, 5; beef extract, 1.5; yeast extract, 1.5 (pH 7.2) at 30 °C. After 12 h, 2% inoculum was transferred to the same medium containing isobutyronitrile (1 g/L) as inducer. Cells were harvested at [a]12 h, [b]24 h and [c]24 h (at 12 h the specific activities of *M. p* and *M. l* are very less) and activity was determined by fluorescence method.[21] [d]The isolates were initially classified according to Gram character,[e] fatty acid profile and other biochemical tests. Finally they were classified using 16S rRNA sequence analysis. The chromosomal DNA of the strains was isolated according to the procedure of Rainey et al.[22] 16S rRNA gene was amplified by two primers 8-27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1500r (5′-AGAAAGGAGGTGATCCAGGC-3′). The amplified DNA fragments of 1.5 kb were separated on 1% agarose gel, eluted from the gel and purified using Quiaquick Gel extraction Kit (Quiagen, Germany). The purified PCR products were sequenced with four forward and three reverse primers namely, 8-27f (5′-AGAGTTTGATCCTGGCTCAG-3′), 357f (5′-CTCCTACGGGAGGCAGCAG-3′), 704f (5′-GTAGCGGTGAAATGCGTAGA-3′), 1114f (5′-GCAACGAGCGCAACC-3′), 685r (5′-TCTACGCATTTCACCGCTAC-3′), 1110r (5′-GGGTTGCGCTCGTTG-3′) and 1500r (5′-AGAAAGGAGGTGATCCAGGC-3′), respectively, of the *E. coli* numbering system. The DNA sequences were determined by the dideoxy chain-termination method using the DYEnamic ET Terminator Kit (Amersham Biosciences, Sweden). Sequencing reaction products were analyzed by capillary electrophoresis on a MegaBase sequencer (Amersham Biosciences, Sweden). The nucleotide sequences obtained were compared with NCBI database using BLASTN. [f,g]$t_{1/2}$ and K_{Deact} were determined from thermostability studies carried out at 30 °C (Fig. 1).

Table 2. Comparison of the isolates base on different kinetic parameters

Strain	Conversion[a] (%)	Selectivity	Ee[b] (%)	E[c]	K_m[d] (mM)	V_{max}[e] (mM/min)	k_{Rxn}[f] (mM/min)
P. putida	29.20	*R*	99.98	>100	2.19	0.502	0.127
M. paraoxydans	15.34	*R*	99.89	>100	4.6	0.45	0.066
M. liquefaciens	16.64	*R*	93.81	32.79	5.3	0.42	0.072

Reaction conditions: (*RS*)-mandelonitrile (2 mM) and whole cells in phosphate buffer (0.1 M, pH 7.0) were shaken at 30 °C for 10 min.
[a]Conversion of nitrile to acid as estimated by RP-HPLC over a period of 10 min.
[b]Ee (%) as estimated on a Chiralcel OD-H column (250×0.46 mm, 5 μm) (Daicel Chemical Industries, Japan) using a mobile phase of hexane–isopropyl alcohol–trifluoro acetic acid, 90:10:0.2. The retention time of *S* and *R* isomer of mandelic acid were 15.5 and 17.5 min, respectively.
[c]The enantiomeric ratios for the acid were calculated from the conversion (C) and the ee as follows: $E = \ln[1 - C(1 + ee)]/\ln[1 + C(1 - ee)]$.
[d,e]K_m and V_{max} were determined from LineWeaver–Burk plots using substrate in the concentration range of 0.25–30 mM.
[f]The reaction rates were measured at a substrate concentration of 2 mM from the linear part of the graph (Fig. 3).

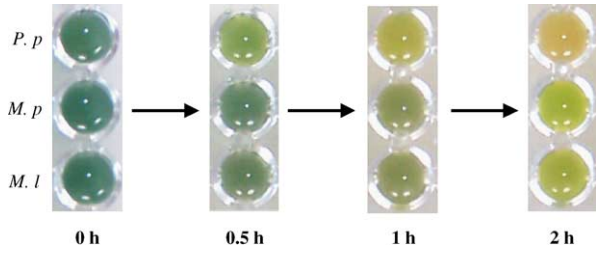

Figure 2. Visualization of nitrile hydrolysis using the pH sensitive indicator based assay. Reaction mixture consisted of bromothymol blue (as indicator), mandelonitrile and cells at a concentration of 0.154, 2 mM and 0.02 mg/μL, respectively, in a total volume of 230 μL. Colour turn over was monitored over a period of 2 h. *P. p*: *P. putida*; *M. p*: *M. paraoxydans*; *M. l*: *M. liquefaciens*.

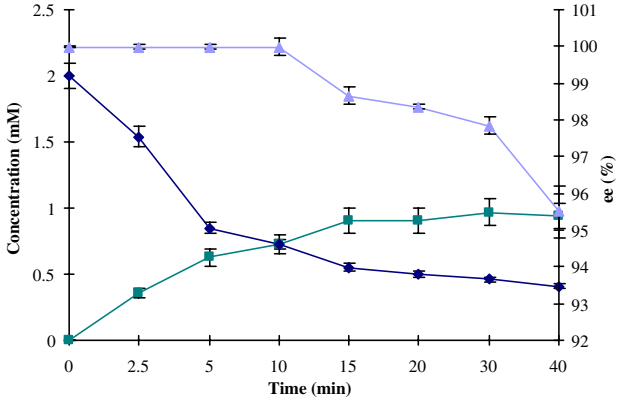

Figure 3. Enantioselective conversion of (*RS*)-mandelonitrile by *P. putida*. ■ Mandelic acid formed, ◆ mandelonitrile remained, ▲ ee (%) of the (*R*)-(−)-mandelic acid formed. The amount of mandelic acid formed and ee (%) was estimated as described earlier.

M. liquefaciens showed lower conversions (Table 2), however the latter two organisms showed comparable conversions to the former when the reactions were allowed to proceed till 4 h (data not shown). All three microorganisms showed high E values. *P. putida* also showed lower K_m and higher V_{max} amongst the short listed organisms, indicating its higher affinity for the substrate. To obtain further high substrate conversion it is suggested to use the purified nitrilases, in order to avoid interferences from other competitive enzymes. In no case was the presence of mandelamide detected in the samples analyzed by RP-HPLC. Thus it can be concluded that microorganisms harbour a nitrilase enzyme,

which is responsible for the enantioselective hydrolysis of racemic mandelonitrile to (*R*)-(−)-mandelic acid.

3. Conclusion

Nitrile hydrolyzing enzymes, especially nitrilases have enormous potential as industrial biocatalysts. In the present study, it has been shown that selection of a substrate analogue, as a sole source of carbon and/or nitrogen during enrichment culture, gives access to bacterial enzyme systems that are highly adapted to the target substrate. All the three microorganisms studied, namely *P. putida*, *M. paraoxydans* and *M. liquefaciens* gave high conversion and ee values. Increasing the biocatalyst concentration in the reaction mixture can further increase the conversion. However maximizing the conversion will in turn reduce the mass of the material that requires racemization/recycling and may also lead to lower ee values. Thus emphasis was given to find a biocatalyst that yields (*R*)-(−)-mandelic acid with ee of more than 98%, a substrate conversion in excess of 40% and *E* value >100.[23] The screen was designed in such a way so as to obtain microorganisms with the above characteristics, the only ones with practical applications.

Final selection of microorganism for the transformation of mandelonitrile to (*R*)-(−)-mandelic acid was based on different characteristics of the microorganism, including its growth rate (μ), conversion and ee values, stability ($t_{1/2}$), reaction rate (k_{Rxn}), etc. Considering all these, *P. putida* seems to be an ideal candidate for further optimization and biocatalysis studies as it has higher growth rate (μ), higher reaction rate (k_{Rxn}) and higher stability ($t_{1/2}$) compared to *M. paraoxydans* and *M. liquefaciens* under the operational conditions. High expression of the concerned enzyme in a rapidly growing microorganism is the primary requirement for the selection of a biocatalyst. Since, *P. putida* has higher μ and attains comparable specific activity within shorter time period (12 h), the fermentation time will be considerably lower for the biocatalyst generation, which will be reflected by process economics. Considering the primary objective of any biocatalytic process is the high degree of substrate conversion in a shortest possible time, the turn over of an enzyme represents a key factor in the concept of an ideal biocatalyst. Also owing to the propensity of mandelonitrile for decomposition into benzaldehyde and HCN, it is necessary to achieve high reaction rates for the enzymatic reactions. As *P. putida* has the faster reaction rate (k_{Rxn} 0.127) than its counterparts, it should be preferred for further studies. This can also be exploited to study fed-batch mode of reaction involving such unstable substrate.

Biocatalysts are inherently labile, therefore their operational stability is of paramount importance for any bioprocess. Poor biocatalyst stability will result in longer process operations (resulting from decreased catalytic efficiency), increased frequency of catalyst replacement and reduced product yield.[7] Since, thermostability is an index of overall biocatalyst stability, it can be concluded that *P. putida*, which has higher stability ($t_{1/2}$ 27.28 h), may lead to a stable biocatalyst under a range of deleterious conditions. According to Arrhenius kinetics, the highest feasible reaction temperature should be selected for a biocatalytic reaction, but such a selection is always constrained by biocatalyst stability. The high stability of nitrilase of *P. putida* should confer more flexibility for a viable process design. Moreover, bioconversions involving hydrophobic substrates such as mandelonitrile would be benefitted by enzymes that exhibit high turn over in organic media. It may therefore be possible to extrapolate the high stability of this novel nitrilase to its high activity and stability in organic solvents for efficient resolution of hydrophobic substrates.

There are few reports on stereoselective arylacetonitrilases applied in the conversion of α-hydroxy aromatic nitriles.[24–26] We have attempted to screen for a biocatalyst, not only from the point of view of reaction kinetics, but also to explore for a suitable enzyme source that couples enhanced reaction rate with its robust character for the ultimate resolution. We therefore conclude that such a multifunctional approach to screening holds the key for successful acquisition of a biocatalyst that ideally meets the requirements of a process. The isolated microorganisms, especially *P. putida* may be a suitable candidate for the production of (*R*)-(−)-mandelic acid from racemic mandelonitrile. Work is in progress in our laboratory to optimize the different physico-chemical parameters for the higher enzyme productivity and characterization of the nitrilase produced by *P. putida*.

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