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ORIGINAL PAPER

Biocatalytic synthesis of (*R*)-(−)-mandelic acid from racemic mandelonitrile by cetyltrimethylammonium bromide-permeabilized cells of *Alcaligenes faecalis* ECU0401

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Abstract The nitrilase from *Alcaligenes faecalis* ECU0401 belongs to the category of arylacetonitrilase, which could hydrolyze 2-chloromandelonitrile, 3,4-dimethoxyphenylacetonitrile, mandelonitrile, and phenylacetonitrile into the corresponding arylacetic acids. To overcome the permeability barrier and prepare whole cell biocatalysts with high activities, permeabilization of *Alcaligenes faecalis* ECU0401 in relation to nitrilase activity was optimized by using cetyltrimethylammonium bromide (CTAB) as permeabilizing agent. The nitrilase activity from *Alcaligenes faecalis* ECU0401 increased 4.5-fold when the cells were permeabilized with 0.3% (w/v) CTAB for 20 min at 25°C and pH 6.5. Consequently, almost all the mandelonitrile was consumed and converted to (*R*)-(−)-mandelic acid with greater than 99.9% enantiomeric excess (*e.e.*) by the CTAB-permeabilized cells. The permeability barrier has been significantly reduced in the hydrolysis of mandelonitrile by using CTAB-permeabilized cells and a dynamic resolution was successfully achieved, giving a 100% theoretical yield of (*R*)-(−)-mandelic acid. Efficient biocatalyst recycling was achieved as a result of cell immobilization in calcium alginate, with a product-to-biocatalyst ratio of 3.82 g (*R*)-(−)-mandelic acid g^{-1} dry cell weight (dcw) cell after 20 cycles of repeated use.

Keywords Nitrilase · *Alcaligenes faecalis* ECU0401 · Mandelonitrile · (*R*)-(−)-Mandelic acid · CTAB permeability

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Introduction

(*R*)-(−)-Mandelic acid and its derivatives are a class of chiral synthons for the production of various pharmaceuticals [1, 9, 16, 25], such as semisynthetic penicillins, cephalosporins, antiobesity agents, and antitumor agents, and is also used as a chiral resolving agent [12–14]. Production of (*R*)-(−)-mandelic acid can be achieved by physicochemical methods [13] as well as by different enzymatic routes. There is a considerable industrial interest in enzymatic conversion of various nitriles owing to the desirability of conducting such conversions under mild conditions that would not alter other labile reactive groups [4, 6, 7, 22, 23, 27]. Biocatalytic hydrolysis of nitriles has been shown to proceed through two distinct pathways: in the presence of a nitrilase (EC 3.5.5.1) [2, 19, 36, 39], nitriles undergo direct bioconversion to the corresponding carboxylic acids and ammonia; whereas a nitrile hydratase (EC 4.2.1.84) [3, 29] catalyzes the hydration of nitriles followed by the biotransformation into the corresponding acids with the aid of an amidase (EC 3.5.14) [34, 38]. The nitrilase-mediated pathway offers significant advantages

over other routes because of the absence of cofactor involvement, cheap starting material in the form of mandelonitrile, and above all the possibility of carrying out a dynamic kinetic resolution which provides theoretically 100% yield of (*R*)-(−)-mandelic acid [37].

Recently we isolated a bacterial strain from soil, *Alcaligenes faecalis* ECU0401 [12, 14], which has nitrilase activity and could enantioselectively transform racemic mandelonitrile into (*R*)-(−)-mandelic acid with an enantiomeric excess (*e.e.*) of greater than 99.9%. Nitrilase of *Alcaligenes faecalis* JM3 and *Alcaligenes faecalis* ATCC 8750 are inducible [37], while the nitrilase of *Alcaligenes faecalis* ECU0401 is a constitutive enzyme. A nitrilase gene *blr3397* from *Bradyrhizobium japonicum* USDA110 was cloned and over-expressed in *E. coli*, which showed higher activity towards the hydrolysis of aliphatic nitriles than that for the aromatic counterparts. But the *e.e.* value and conversion of (*S*)-(−)-mandelic acid were 36 and 44%, respectively [39]. Kaul reported that the *e.e.* values of (*R*)-(−)-mandelic acids were very high at the start of the reaction with three bacterial isolates, but dropped as the reaction proceeds and more product formation occurred [18]. In our case, the *e.e.* value of (*R*)-(−)-mandelic acid was kept above 99.9%. However, the yield of (*R*)-(−)-mandelic acid is quite low (≤55.0%) in our case. The objective of the present study was to enhance the catalytic efficiency of *Alcaligenes faecalis* ECU0401.

Materials and methods

Chemicals

Cetyltrimethylammonium bromide (CTAB) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other reagents and chemicals used were also from commercial sources and of reagent grade.

Culture medium

Solid medium: glycerol 10 g/L, peptone 10 g/L, yeast extract 5 g/L, KH_2PO_4 2 g/L, NaCl 1 g/L, $MgSO_4$ 0.2 g/L, agar 15% g/L, pH 7.0.

Fermentation medium: 10 g/L sodium acetate, 10 g/L peptone, 5 g/L yeast extract, 3 g/L KH_2PO_4, 1.5 g/L NaCl, 0.3 g/L $MgSO_4$, and 0.08 g/L $CuSO_4$, pH 6.0.

Microorganism, growth, and bioconversion conditions

Alcaligenes faecalis ECU0401 having nitrilase activity was isolated from soil [14], and this strain has been deposited at the China General Microbiological Culture Collection Center with an accession number of CGMCC 2009. No nitrile hydratase activity was detected in the hydrolysis of nitriles [12, 21].

The strain, after being grown on solid medium for 48 h, was first precultivated in 5 mL growth medium (solid medium without agar) for 12 h, then inoculated into 1,000-mL Erlenmeyer flasks containing 195 mL of the growth medium and shaken at 30°C and 160 rpm. After 48 h of cultivation, cells were harvested from the culture broth by centrifugation (12,000×*g*, 4°C, 15 min), washed, and resuspended in the potassium phosphate buffer (100 mM, pH 6.5).

Hydrolysis of racemic mandelonitrile was determined by using the resuspended whole cells of *Alcaligenes faecalis* ECU0401. The reaction, consisting of 20 mM of substrate and 1.0 g wet weight (ca. 0.15 g dry cell weight (dcw)) of whole cells in 10 mL phosphate buffer (pH 6.5, 100 mM), was carried out at 30°C and 160 rpm. The bioconversion process was monitored with high-performance liquid chromatography (HPLC) by withdrawing 0.2 mL of samples at fixed time intervals. The reaction was stopped by adding 20 μL of 2 M H_2SO_4 to a 180-μL sample followed by centrifugation. The clear supernatant, diluted with buffer when necessary, was assayed for substrate and product concentrations by HPLC.

Analytical methods

The amounts of mandelic acid, benzaldehyde, and mandelonitrile were assayed by HPLC (Shim-pack VP-ODS, Shimadzu Co., Japan) at a flow rate of 0.8 mL/min, with a mixed eluent of phosphate buffer (10 mM, pH 4.8) and methanol (87:13, v/v). The UV absorption at 215 nm was measured. The retention times for mandelic acid, benzaldehyde, and mandelonitrile were 8.1, 26.9, and 33.3 min, respectively. The enantiomeric excess (*e.e.*, %) of (*R*)-(−)-mandelic acid was also determined with HPLC using a chiral column (Chiralcel OD-H, Daicel Co., Japan) which was eluted with hexane/isopropanol/trifluoroacetic acid (90:10:0.2, v/v/v, 0.8 mL/min) and detected at 228 nm. The retention times for (*S*)-(+)-mandelic acid and (*R*)-(−)-mandelic acid were 11.2 and 13.0 min, respectively.

Cell viability was determined by the flow cytometry method [8, 15]. After a sample was diluted to 1×10^6 cells/mL with phosphate buffer, 300 μL was mixed with 2 μL propidium iodide (PI, Sigma) solution (1 mg/mL) and analyzed within 10 min. This dye could enter only cells with leaky membranes and therein bind to DNA, exhibiting fluorescence with excitation at 488 nm and an emission maximum at 660 nm. Cells were analyzed on a FACScalibur (Becton–Dickinson, Franklin Lakes, NJ, USA) with a 488-nm argon laser and a 660-nm diode laser. Using phosphate buffer as the sheath fluid, we measured 1×10^4 cells per analysis. For viability measurements,

cellular autofluorescence was measured through a 530/30-BP filter [15].

Nitrilase assays

Nitrilase activity in the whole cells was measured by using the ammonia release assay of Fawcett and Scott [10]. One unit of activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol ammonia per min at 30°C under standard assay conditions.

Treatment of cells with CTAB

Cells were harvested during stationary phase then separated by centrifugation at 12,000×*g* and 4°C for 15 min. The whole cells were resuspended in phosphate buffer (100 mM, pH 6.5) to a final concentration of 0.15 g dcw/10 mL; CTAB was added to a final concentration from 0.1 to 0.5% (w/v), and then stirred gently on a rotary shaker (160 rpm) at different temperatures for different times. After this, the cells were recentrifuged and washed twice with the same buffer, and analyzed for their nitrilase activity.

Treatment of cells by sonication

The centrifuged cells were resuspended in phosphate buffer (100 mM, pH 6.5) to a final cell concentration of 0.15 g dcw/10 mL. Cell wall was disrupted with the ultrasonic cell disruptor (Ningbo Scientz Biotechnology, JY9Z-II, China), which was at 400 W for 100 times (working 3 s and intervals 7 s as 1 cycle), with cooling in an ice-water bath. The treated sample was observed through a microscope (10 × 100, XSP-10C, Shanghai Optical Instrument, China) after being diluted appropriately and stained with Crystal violet; the sample was found be disrupted entirely. The cell debris was removed by centrifugation (20,000×g, 30 min, 4°C), and the supernatant obtained was designated as cell-free extract. The reaction, consisting of 20 mM of substrate and 10 mL cell-free extract (pH 6.5, 100 mM), was carried out at 30°C and 160 rpm. The bioconversion process was monitored with HPLC.

Hydrolysis of various nitriles by CTAB-permeabilized cells of *Alcaligenes faecalis* ECU0401

Hydrolysis of various nitriles (acrylonitrile, acetonitrile, 2-chloromandelonitrile, diaminomaleonitrile, 2,3-dicyanopyrazine, 3,4-dimethoxyphenylacetonitrile, glycolonitrile, iminodiacetonitrile, mandelonitrile, and phenylacetonitrile) was determined by using the CTAB-permeabilized cells of *Alcaligenes faecalis* ECU0401. The reactions were performed at 30°C and 160 rpm in 10 mL potassium phosphate buffer (pH 6.5, 100 mM) with 0.15 g dcw/10 mL, and the substrate concentrations were 20 mM, respectively.

Immobilization of cells and repeated batch biosynthesis of (*R*)-(−)-mandelic acid by immobilized cells

The cell suspension (5.0% dcw) obtained after suspending the CTAB-permeabilized cells in buffer was added to sodium alginate solution, and the slurry containing 2% (w/v) Na-alginate was added dropwise to an ice-cold solution of calcium chloride (100 mM) 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 (approximately 2.5-mm diameter) were washed with Tris–HCl buffer (100 mM, pH 7.0) to remove excess $CaCl_2$ and finally suspended in the same buffer for further biocatalytic reaction.

To assess the reuse potential of the alginate entrapped cells, repeated batch transformation of racemic mandelonitrile were carried out under the same experimental conditions described for the first cycle. The reaction, consisting of 20 mM of substrate racemic mandelonitrile and 10.0 g of immobilized beads containing 2.0% (dcw) cells in 100 mL Tris–HCl buffer (100 mM, pH 7.0), was carried out for 12 h at 30°C and 160 rpm. After each cycle of complete conversion, the beads were washed with physiological saline (0.85% NaCl, w/v) and transferred into a fresh 100 mM Tris–HCl buffer.

Results and discussion

Investigation into the reason for the low yield of (*R*)-(−)-mandelic acid

In the hydrolysis of mandelonitrile by the resting cells of *Alcaligenes faecalis* ECU0401, (*R*)-(−)-mandelic acid was obtained in a low yield of 55.0% [12] after 24 h without any detectable amount of mandelamide as a possible by-product, and no degradation of the product (*R*)-(−)-mandelic acid was also detected. The hydrolysis product (*R*)-(−)-mandelic acid might accumulate intracellularly. In this way, mandelic acid could be a potentially toxic compound. This toxicity could have been reduced by destroying the permeability barrier. Thus, the whole cells used for the biotransformation for 24 h with the phosphate buffer containing 11.0 mM (*R*)-(−)-mandelic acid were destroyed by the ultrasonic cell disruptor in a cold ice-water bath. No significant accumulation of (*R*)-(−)-mandelic acid was found intracellularly. Most likely, the low yield of (*R*)-(−)-mandelic acid results from the toxicity to the cells of

Alcaligenes faecalis ECU0401 caused by mandelonitrile, mandelonitrile's spontaneous degradation products (benzaldehyde and HCN), and hydrolysis product (*R*)-(−)-mandelic acid, or the permeability barrier of mandelonitrile.

The toxicity to the cells of Alcaligenes faecalis ECU0401 caused by substrate and product

The biotransformation activity in the presence of various concentrations of benzaldehyde, HCN, and (*R*)-(−)-mandelic acid was evaluated (Fig. 1). No obvious inhibition was observed with concentrations of benzaldehyde, HCN, and (*R*)-(−)-mandelic acid from 1 to 20 mM. However, benzaldehyde exerted a significant effect on biotransformation activity at high concentration: at 50 mM, benzaldehyde and (*R*)-(−)-mandelic acid resulted in 23.1 and 13.2% inhibition, respectively. We concluded that low concentrations of benzaldehyde, HCN, and (*R*)-(−)-mandelic acid did not significantly inhibit the nitrilase activity of *Alcaligenes faecalis* ECU0401.

The toxicity of mandelonitrile to cells of *Alcaligenes faecalis* ECU0401 was also determined by measuring the cell viability with a flow cytometry. Before detecting cell viability, the method was qualified by using samples from fresh shake flask cultures without and with heat treatment over a flame (to kill the cells) [15]. It was demonstrated that a clearly distinguishable population of PI-stained cells appeared in the heat-damaged sample. Thus, it was easier to draw the gating regions to discriminate between viable and dead cells (Scheme 1). As shown in Scheme 1 and Table 1, the death rate (2.34%) with addition of mandelonitrile for 14 h was quite low and close to that with addition of ethanol as control for 14 h (2.04%), and residual nitrilase activity of mandelonitrile-treated cells reached 92.7%, indicating that low toxicity of mandelonitrile to the cells of *Alcaligenes faecalis* ECU0401 was detected by measuring the cell viability with flow cytometry.

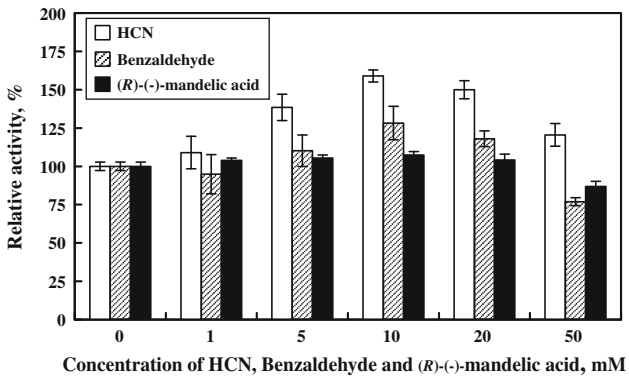
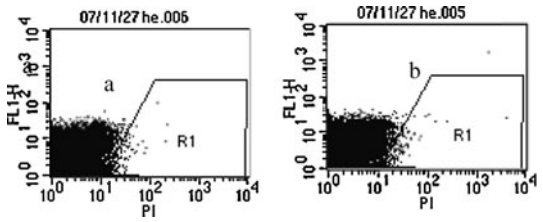


Fig. 1 Effects of different concentrations of HCN, benzaldehyde, or (*R*)-(−)-mandelic acid (*closed columns*) on the biotransformation activity by *Alcaligenes faecalis* ECU0401. The enzyme activity measured without addition of HCN, benzaldehyde, or (*R*)-(−)-mandelic acid was taken as 100%. All experiments were performed in triplicate



Scheme 1 The change of cell viability in *Alcaligenes faecalis* ECU0401 measured by flow cytometry

Table 1 The change of cell viability in *Alcaligene faecalis* ECU0401 measured by flow cytometry

Entry	Death rate, %	Residual activity, %
Control[a]	2.04 ± 0.07	100 ± 1.5
Sample[b]	2.34 ± 0.03	92.7 ± 2.3

Residual enzyme activity measured under the control conditions was taken as 100%. All experiments were performed in triplicate

[a] 0.15 g (dcw) of harvest cells was respended in 10 mL phosphate buffer (pH 6.5, 100 mM), and 20 μL of ethanol was added into buffer. The cells were shaken at 30°C and 160 rpm for 14 h

[b] 0.15 g (dcw) of harvest cells was respended in 10 mL phosphate buffer (pH 6.5, 100 mM), and 20 μL of racemic mandelonitrile (final concentration, 20 mM) with cosolvent of ethanol was added into buffer. The cells were shaken at 30°C and 160 rpm for 14 h

Production of (R)-(−)-mandelic acid from racemic mandelonitrile by cell-free extract

In order to verify if the permeability barrier affects the production of (*R*)-(−)-mandelic acid, cell-free extract achieved by sonication was used to hydrolyze the racemic mandelonitrile (Fig. 2). A yield of 94.0% was obtained after 24 h. We concluded that, in whole cell biocatalysis, the permeability barrier affects the production of (*R*)-(−)-mandelic acid.

On the basis of these experiments, we found that low concentrations of mandelonitrile, benzaldehyde, HCN, and (*R*)-(−)-mandelic acid (0–20 mM) did not affect the nitrilase activity, and the permeability barrier might cause the low yield of (*R*)-(−)-mandelic acid.

Hydrolysis of mandelonitrile by CTAB-permeabilized cells

In order to overcome the permeability barrier, anionic, cationic, or nonionic detergents could be used to permeabilize the whole microbial cells [26, 30, 31, 33, 35]. The

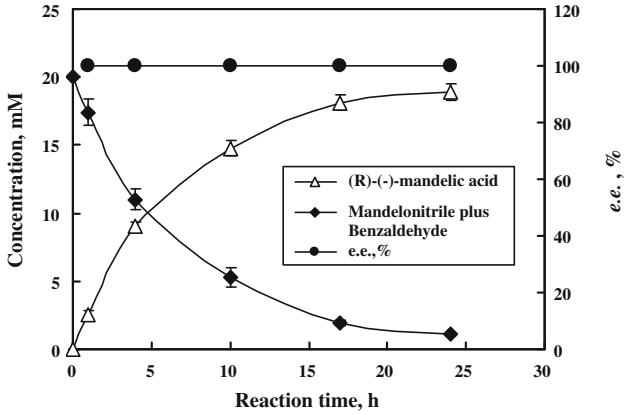


Fig. 2 Time course of (*R*)-(−)-mandelic acid production from racemic mandelonitrile by cell-free extract. All experiments were performed in triplicate

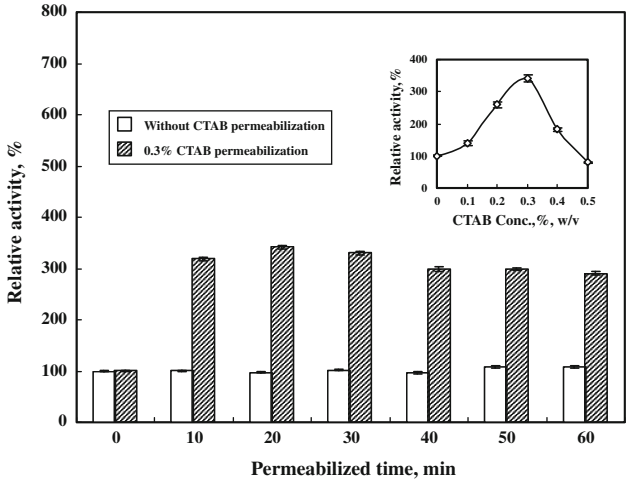


Fig. 3 Effects of different concentrations of CTAB and permeabilization times on cell permeabilization. At 30°C, specific activity of nitrilase in *Alcaligenes faecalis* ECU0401 without CTAB permeabilization was 1,010.4 μmol min^{-1} g^{-1}. All experiments were performed in triplicate

detergent-treated whole cells have been studied as a source of intracellular enzymes instead of expensive isolated enzymes. Nagalakshmi and Pai treated cells with CTAB and the penicillin acylase activity increased about twofold [24]. Therefore, we investigated the use of CTAB as a potential detergent to permeabilize the whole cells of *Alcaligenes faecalis* ECU0401.

Optimization of CTAB permeabilization

The effect of CTAB concentration on the cell permeabilization process was investigated (Fig. 3), in which the CTAB concentration ranged from 0.1 to 0.5% (w/v). A 341% relative increase of biotransformation activity was achieved when 0.3% (w/v) CTAB was used to permeabilize the whole cells. Less nitrilase activity at low concentrations of CTAB might be due to the amount of the agent being insufficient for effective permeabilization. The decrease in nitrilase activity at higher concentration of CTAB might be attributed to the leakage of the enzyme from the cells or cell lysis. Obviously, the permeabilization time also had profound effects on enzyme activity. Ten min of permeabilization time might be insufficient for effective permeabilization, and the hydrolysis rate exhibited a maximum after being permeabilized with 0.3% (w/v) CTAB for 20 min. The slow decrease in nitrilase activity with prolonging the permeabilization time might be attibuted to the leakage of the enzyme from the cells or cell lysis. Different temperatures were employed to investigate this parameter's effect on cell permeability process (Table 2). The best result was obtained at 25°C, which was 130% of total enzyme activity at 30°C. When the permeabilized temperature was above 25°C, the numerical value of the relative enzyme activity declined, which reached only 95.9% at 35°C. If the permeabilized temperature increased to 50°C, almost no enzyme activity was detected. Therefore, the optimal CTAB concentration 0.3% (w/v), permeabilization time 20 min, and permeabilized temperature 25°C were employed for the following experiments.

Production of (R)-(−)-mandelic acid from racemic mandelonitrile by CTAB-permeabilized cells

To determine the effectiveness of using CTAB-pretreated cells in the production of (*R*)-(−)-mandelic acid from racemic mandelonitrile, the time course of the reaction was investigated. Because the racemic mandelonitrile, a cyanohydrin, is in equilibrium in aqueous solution (with benzaldehyde and HCN), the amount of substrate was taken to be the total amount of mandelonitrile and benzaldehyde. The amount of (*R*)-(−)-mandelic acid produced corresponded to the decrease in the amount of the substrate (Fig. 4), whereas no mandelamide was detected at any time during the reaction, evidence that *Alcaligenes faecalis* ECU0401 had only the nitrilase for mandelonitrile. The yield of (*R*)-(−)-mandelic acid against the racemic substrate was 94.5% after 24 h. The reason for this may be that residual (*S*)-mandelonitrile which was unreactive to the nitrilase was spontaneously racemized because of the chemical equilibrium ((*S*)-mandelonitrile $\rightleftharpoons$ benzaldehyde + HCN $\rightleftharpoons$ (*R*,*S*)-mandelonitrile) and then used as the substrate for the nitrilase, thereby being consumed and converted to (*R*)-(−)-mandelic acid. This could be a reason why the yield increases from 55.0 to 94.5% using racemic mandelonitrile as substrate. It is possible that a racemase catalyzes the racemization of (*S*)-mandelonitrile, or it was spontaneously racemized because of the chemical equilibrium. In order to determine if the whole cells contained

Table 2 Effect of temperature on cell permeabilization

Treatment temperature, °C	Relative nitrilase activity, %	*e.e.*, %
20	93.8 ± 0.12	>99.9
25	130 ± 0.16	>99.9
30	100 ± 0.18	>99.9
35	95.9 ± 0.14	>99.9
50	ND	–

After being permeabilized for 20 min with 0.3% (w/v) of CTAB at 30°C, specific activity of nitrilase in *Alcaligenes* sp. ECU0401 was 3,443.8 μmol min^{-1} g^{-1}, and the enzyme activity measured was taken as 100%. All experiments were performed in triplicate

ND not detected

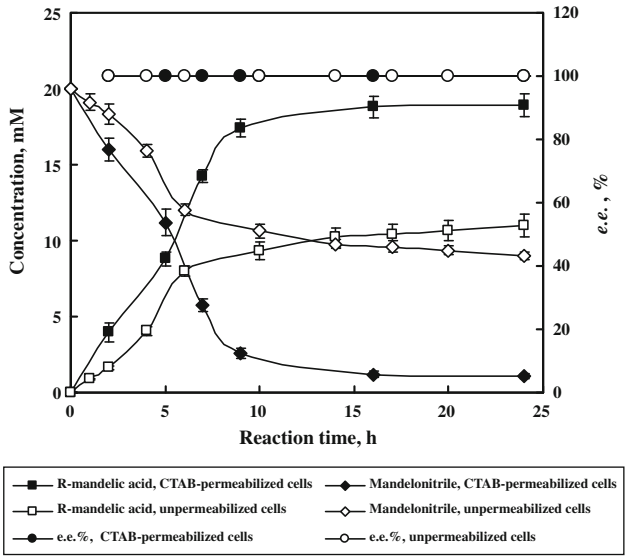


Fig. 4 Time course of (*R*)-(−)-mandelic acid production from racemic mandelonitrile by CTAB-permeabilized and unpermeabilized *Alcaligenes faecalis* ECU0401. All experiments were performed in triplicate

racemase, racemic α-acetylmandelonitrile (20 mM) was used as substrate, and (*R*)-(−)-α-acetylmandelic acid (*e.e.* > 99.0%) was obtained in 49.5% yield after 24 h. After prolonged reaction time, the concentration of remaining (*S*)-α-acetylmandelonitrile did not change after 36 h, keeping constant at 10.0 mM, without any detectable amount of amide as a possible by-product. These experiment indicated that no racemase catalyzed the racemization of (*S*)-nitrile. At the point of spontaneous racemization of mandelonitrile, it was spontaneously racemized because of the chemical equilibrium [32, 37].

The production of (*R*)-(−)-mandelic acid from benzaldehyde and HCN at pH 6.5 was studied to test the mechanism (Scheme 2). (*R*)-(−)-Mandelic acid was produced in the same way when benzaldehyde plus HCN was used as the substrate (Fig. 5). A high reaction yield of 92.5% was also obtained for (*R*)-(−)-mandelic acid production by initiating the reaction directly from the mixture of benzaldehyde and HCN, indicating that the benzaldehyde and HCN had been converted to racemic mandelonitrile because of chemical equilibrium and then utilized as the substrate of bioconversion, and high *e.e.* value (>99.9%) of (*R*)-(−)-mandelic acid was obtained. In conclusion, an efficient CTAB permeabilization method for reducing permeability barrier was developed, and the yield of the (*R*)-(−)-mandelic acid was significantly improved.

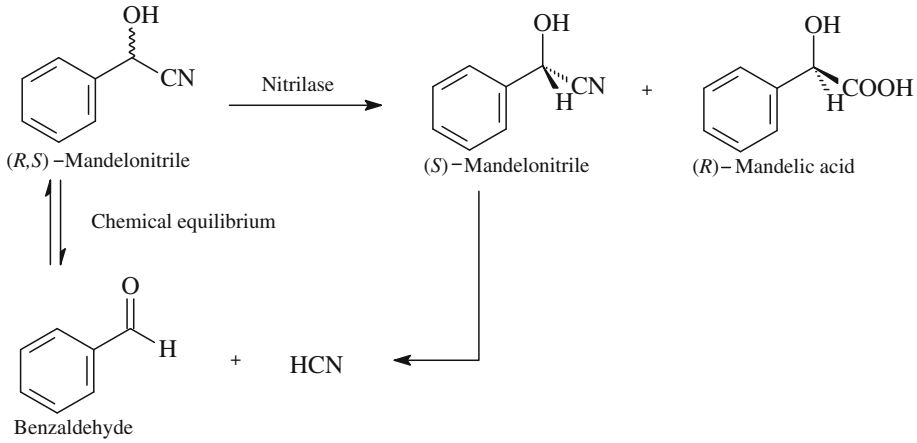
Hydrolysis of various nitriles by CTAB-permeabilized cells

The ability of CTAB-permeabilized cells to catalyze the hydrolysis of various nitriles was examined (Table 3). Nitrilase from *Alcaligenes faecalis* ECU0401 had broad substrate specificity in hydrolyzing both aliphatic and aromatic nitriles. Using arylacetonitriles as substrate, high nitrilase activities were obtained, and the activity for the hydrolysis of phenylacetonitrile was higher than those for 3,4-dimethoxyphenylacetonitrile, mandelonitrile, and 2-chloromandelonitrile. However, the cells displayed relatively lower activities towards aliphatic nitriles, indicating that the nitrilase from *Alcaligenes faecalis* ECU0401 belongs to the category of arylacetonitrilase [20]. To our knowledge, phenylacetic acid and 3,4-dimethoxyphenylacetic acid are also important intermediates in the synthesis of pharmaceutical preparations [5, 28], In our case, CTAB-permeabilized cells of *Alcaligenes faecalis* ECU0401 could effectively synthesize these arylacetic acid compounds from the corresponding nitriles, and phenylacetic acid and 3,4-dimethoxyphenylacetic acid could be obtained in 100% yield after 20 h. Based on the above data, CTAB-permeabilized cells of *Alcaligenes faecalis* ECU0401 show promise in catalyzing the hydrolysis of arylacetonitriles.

Efficient biocatalyst recycling

Immobilized cells are preferred as in this form they can be recycled, thereby reducing production costs. Entrapment seemed to be a better choice for immobilization [1, 4, 12, 17], and immobilization of *Alcaligenes faecalis* ECU0401 cells in calcium alginate beads was used for further racemic mandelonitrile hydrolysis. To investigate the effect of recycling on the degree of mandelonitrile conversion, biotransformation reaction was carried out both with free cells and alginate-entrapped cells in batch mode. Each time, the hydrolytic reaction was carried out for 12 h at 30°C. With the increase in number of cycles, more than 60.0% conversion was obtained with free CTAB-permeabilized cells after 5 cycles. However, more than 90.0% conversion with *e.e.* greater than 99.9% was obtained with immobilized CTAB-permeabilized cells even after

Scheme 2 Enantioselective hydrolysis of manelonitrile to (*R*)-(−)-mandelic acid by nitrilase



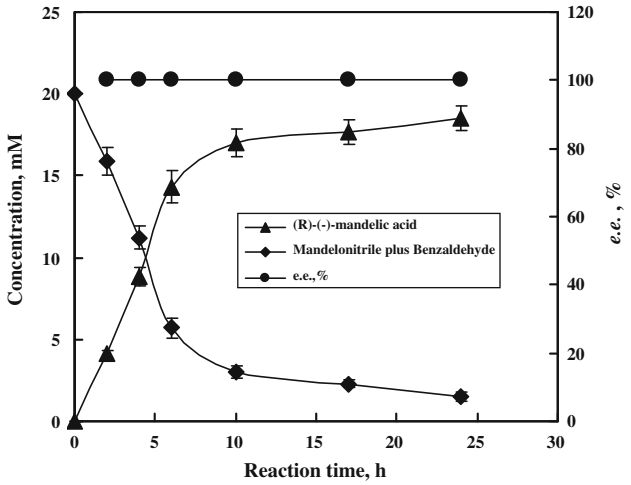


Fig. 5 Time course of (*R*)-(−)-mandelic acid production from benzaldehyde plus HCN by CTAB-permeabilized *Alcaligenes faecalis* ECU0401. All experiments were performed in triplicate

20 cycles (Fig. 6). The biocatalyst productivity of 3.82 g mandelic acid g^{-1} dcw cell (0.57 g mandelic acid g^{-1} wet cell) and the volumetric productivity of 0.24 g mandelic acid L^{-1} h^{-1} were achieved by using immobilized cells. Similar results were reported by Banerjee et al. whose mandelic acid was formed in *e.e.* 98.8% by immobilized cells, and the volumetric productivity (g mandelic acid L^{-1} h^{-1}) and the catalyst productivity (g mandelic acid g^{-1} wet cell) were 0.49 and 0.39, respectively [1]. It appeared reasonable to suggest, therefore, that these results proved that cells immobilized in calcium alginate beads could be reused.

The reaction mixture collected from the 20 batch cycles was adjusted to pH 8.5 with 2 mol/L NaOH, and the unreacted mandelonitrile and produced benzaldehyde were extracted with dichloromethane. The water layer was acidified with 2 mol/L H_2SO_4 to pH 1.0 and extracted with an equal volume of ether for 3 times. The extract obtained was concentrated under reduced pressure and yielded

Table 3 Hydrolysis of various nitriles by CTAB-permeabilized cells

Substrate	Relative activity[a], %	Conversion, %	*e.e.*, %
Iminodiacetonitrile	ND	ND	–
2,3-Dicyanopyrazine	ND	ND	–
Acetonitrile	4.0 ± 0.54	18.4 ± 0.48	–
Glycolonitrile	4.6 ± 0.46	100 ± 0.0	–
Acrylonitrile	8.6 ± 0. 37	70.8 ± 1.26	–
Diaminomaleonitrile	12.2 ± 0.78	46.3 ± 0.54	–
2-Chloromandelonitrile	12.6 ± 0.44	96.1 ± 0.41	>99.9
3,4-Dimethoxyphenyl-acetonitrile	79.2 ± 1.32	100 ± 0.0	–
Mandelonitrile	100.0 ± 1.73	94.5 ± 0.32	>99.9
Phenylacetonitrile	152.3 ± 3.17	100 ± 0.0	–

Hydrolysis of various nitriles was determined by using the resting cells of *Alcaligenes faecalis* ECU0401. The reactions were performed for 20 h at 30°C and 160 rpm in 100 mL potassium phosphate buffer (100 mM, pH 6.5) with 10.0 g wet weight of resting cells, and the substrate concentrations were 20 mM. The conversions were assayed by HPLC with a C18 column, and the *e.e.* was determined by HPLC with Chiralcel OD-H column. All experiments were performed in triplicate

ND not detected, – not determined

[a] The enzyme activity measured by using mandelonitrile as substrate was taken as 100%

5.59 g of crystals. A white powder of the product with a total yield of 91.0% was recrystallized from benzene. The (*R*)-(−)-mandelic acid formed has an *e.e.* of greater than 99.9% (Scheme 3). The specific rotation, $[\alpha]_D^{25}$, was −155° (c = 1.0, H_2O) [Lit. [37]: $[\alpha]_D^{25}$ −155° (c = 1.0, H_2O)]. ^{1}H NMR (400 MHz, DMSO, δ/ppm): 7.26–7.40 (m, 5H), 4.99 (s,1H).

Conclusions

Biological processes for the production of (*R*)-(−)-mandelic acid are often preferred to chemical processes [11]

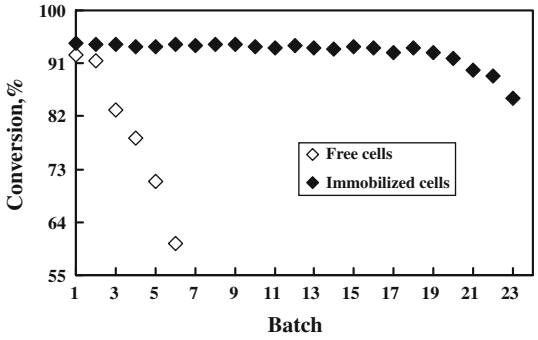
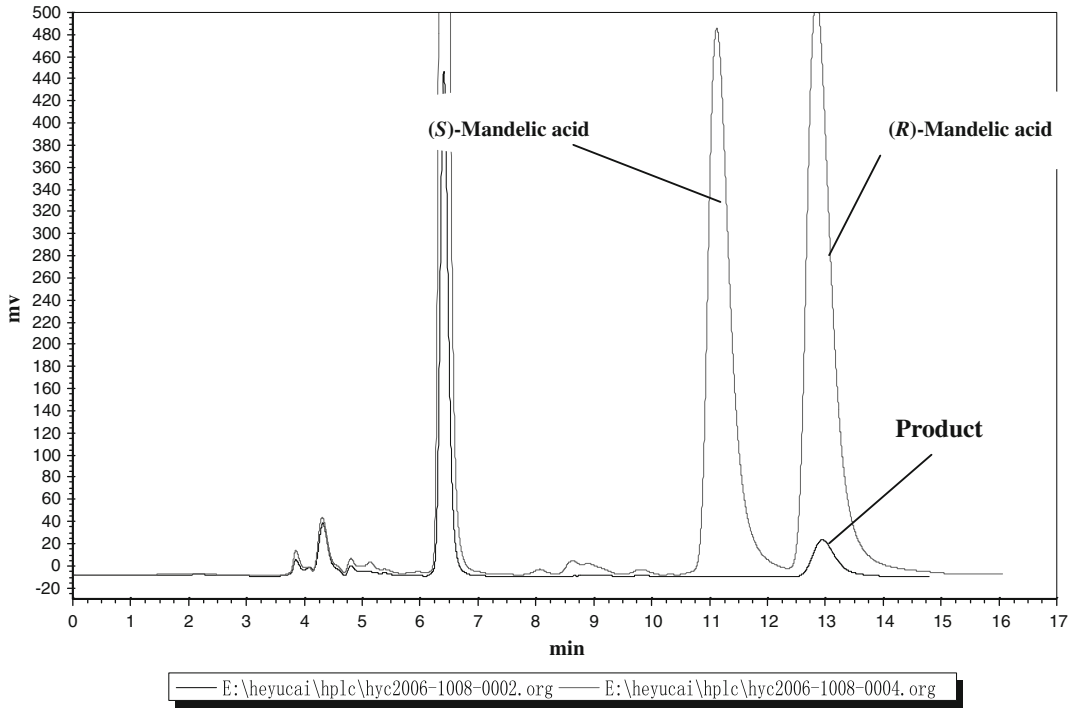


Fig. 6 Batch numbers of immobilized cells and free cells hydrolyzing mandelonitrile. The reaction, consisting of 20 mM of substrate racemic mandelonitrile and 10.0 g of immobilized beads in 100 mL Tris–HCl buffer (100 mM, pH 7.0), was carried out for 12 h at 30°C and 160 rpm. After each cycle of complete conversion, the beads were washed with physiological saline (0.85% NaCl, w/v) and transferred into a fresh Tris–HCl buffer (100 mM, pH 7.0)

since here actions are run at ambient temperature, do not require strongly acidic or basic reaction conditions, and produce the desired product with high selectivity at high conversion. *Alcaligenes faecalis* ECU0401 having nitrilase activity was isolated from soil. In the hydrolysis of racemic mandelonitrile substrate, no degradation of the product (*R*)-(−)-mandelic acid, no mandelamide as a possible by-product, and no toxicity of substrate to cells were detected. And low concentration mandelonitrile benzaldehyde, HCN, and (*R*)-(−)-mandelic acid did not significantly affect the nitrilase activity. However, due to the permeability barrier, (*R*)-(−)-mandelic acid was obtained in a low yield of 55.0% after 24 h. In order to overcome the permeability barrier, cetyltrimethylammonium bromide (CTAB) was used to permeabilize the whole cells of *Alcaligenes faecalis* ECU0401, and permeabilization of *Alcaligenes faecalis* ECU0401 in relation to nitrilase activity was optimized by using CTAB as permeabilizing agent. An efficient CTAB permeabilization method for reducing permeability barrier and enhancing the yield of the (*R*)-(−)-mandelic acid was thus developed. The nitrilase activity from *Alcaligenes faecalis* ECU0401 increased 4.5-fold when cells were permeabilized with 0.3% (w/v) CTAB for 20 min at 25°C and pH 6.5. Consequently, the permeability barrier has been significantly reduced in the hydrolysis of mandelonitrile by using CTAB-permeabilized cells and a dynamic resolution was successfully achieved giving a 100% theoretical yield of (*R*)-(−)-mandelic acid. When the permeabilized cells were immobilized in calcium alginate beads, the immobilized beads had better stability than free cells. Efficient reusability of the biocatalyst up to 20 batches was achieved by immobilization. The use of stabilized, alginate-immobilized *Alcaligenes faecalis* ECU0401 for the synthesis of (*R*)-(−)-mandelic acid may be a viable alternative in future applications; furthermore, continuous and larger-scale production of (*R*)-(−)-mandelic acid by the aid of these immobilized beads in different reactors is currently under progress in our lab. Moreover, the nitrilase from



Scheme 3 Product analysis by HPLC with Chiralcel OD-H column

Alcaligenes faecalis ECU0401 belongs to the category of arylacetonitrilase, which could hydrolyze 2-chloromandelonitrile, 3,4-dimethoxyphenylacetonitrile, mandelonitrile, and phenylacetonitrile into the corresponding arylacetic acids. The arylacetonitrilase gene from *Alcaligenes faecalis* ECU0401 was cloned (data not shown). Screening of the recombinant *E. coli* strain with much higher arylacetonitrilase activity, excellent enantioselectivity, and strong substrate/product concentration tolerance is under progress.

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