APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1991, p. 3028–3032
0099-2240/91/103028-05$02.00/0

Vol. 57, No. 10

Production of *R*-(−)-Mandelic Acid from Mandelonitrile by *Alcaligenes faecalis* ATCC 8750

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Received 30 April 1991/Accepted 30 July 1991

***R*-(−)-Mandelic acid was produced from racemic mandelonitrile by *Alcaligenes faecalis* ATCC 8750. Ammonium acetate or L-glutamic acid as the carbon source and *n*-butyronitrile as the inducer in the culture medium were effective for bacterial growth and the induction of *R*-(−)-mandelic acid-producing activity. The *R*-(−)-mandelic acid formed from mandelonitrile by resting cells was present in a 100% enantiomeric excess. *A. faecalis* ATCC 8750 has an *R*-enantioselective nitrilase for mandelonitrile and an amidase for mandelamide. As *R*-(−)-mandelic acid was produced from racemic mandelonitrile in a yield of 91%, whereas no *S*-mandelonitrile was left, the *S*-mandelonitrile remaining in the reaction is spontaneously racemized because of the chemical equilibrium and is used as the substrate. Consequently, almost all the mandelonitrile is consumed and converted to *R*-(−)-mandelic acid. *R*-(−)-Mandelic acid was also produced when benzaldehyde plus HCN was used as the substrate.**

R-(−)-Mandelic acid is an optical resolving reagent and the source of such pharmaceuticals as semisynthetic cephalosporins. The optically active acid is prepared by optical resolution of the racemate with chiral amines (3) or enzymatically with esterase (15), glyoxalase (16), and (*R*)-mandelate dehydrogenase (20). The enzymatic method has not been used for industrial production because the coenzyme and enzyme sources cannot be produced economically. We therefore investigated a new production process that uses the action of microorganisms on racemic mandelonitrile.

Some microbes convert nitrile compounds to the corresponding acids by the action of nitrilase or of nitrile hydratase plus amidase. L-α-Hydroxyisovaleric acid and L-α-hydroxyisocaproic acid (2) as well as *S*-(+)-ibuprofen (19) have been produced from the corresponding nitriles by various microorganisms, but little is known about the production of optically active mandelic acid by nitrile-hydrolyzing enzymes.

We report the screening of microorganisms that enantioselectively hydrolyze racemic nandelonitrile to produce optically active mandelic acid. The microorganism *Alcaligenes faecalis* ATCC 8750 was studied in depth with regard to its ability to produce *R*-(−)-mandelic acid from mandelonitrile or benzaldehyde and HCN.

MATERIALS AND METHODS

Chemicals. Mandelonitrile and mandelic acid were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Mandelamide was synthesized at 100°C for 4 h by the reaction of ethyl mandelate with ammonia in ethanol and then crystallized as described by McKenzie and Wren (14). The analytical data matched those reported previously (17).

Peptone and yeast extract D-3 were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). All the other chemicals used were obtained from commercial sources.

Screening for mandelic acid-producing strains. Microorganisms were grown aerobically at 32°C for 1 to 2 days in a rich medium that contained (per liter) 10 g of glucose, 5 g of yeast extract D-3, 5 g of peptone, 5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 30 mg of $FeSO_4 \cdot 7H_2O$, 1 g of NaCl, and 1 g of isobutyronitrile (pH 7.2). Harvested cells were centrifuged and suspended in 0.1 M potassium phosphate buffer (pH 8.0). The reaction mixture used to screen the mandelic acid-producing strains contained 100 μmol of potassium phosphate buffer (pH 8.0), 2 mg of mandelonitrile or mandelamide as the substrate, and the suspended cells (final dry weight, 2 to 110 mg) in a total volume of 1.0 ml. The reaction was performed at 32°C for 4 h with reciprocal shaking (350 strokes per min) and then terminated by centrifugation at 18,000 × *g* for 10 min. The amount of mandelic acid present in the supernatant was determined as described below.

Isolation of *R*-(−)-mandelic acid. *A. faecalis* ATCC 8750 was cultured at 32°C for 20 h in medium that contained (per liter) 10 g of ammonium acetate, 5 g of yeast extract D-3, 5 g of peptone, 5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 30 mg of $FeSO_4 \cdot 7H_2O$, 1 g of NaCl, and 3 g of *n*-butyronitrile (pH 7.2). The cells (3.0 g [dried]) were collected by centrifugation and suspended in 2,240 ml of 0.1 M potassium phosphate buffer (pH 8.0). A 520-mg portion of mandelonitrile was added to 100 ml of the cell suspension, after which the mixture was incubated at 32°C with stirring (120 rpm). The cells were removed by centrifugation after 4 h. The pH was adjusted to 8.5 with 2 N NaOH, and the supernatant was washed with 100 ml of ether. After the pH of the water layer had been adjusted to 1.5 with 6 N HCl, the desired product was extracted with 100 ml of ether. The extract obtained was concentrated under reduced pressure and yielded 534 mg of crystals. These were recrystallized from benzene (510 mg).

Analytical methods. The amounts of mandelic acid, mandelamide, mandelonitrile, and benzaldehyde were assayed by analytical high-pressure liquid chromatography (Tosoh CCPM system equipped with a Unisil Pack 5C18-100A column [Gasukuro Kogyo Inc., Tokyo, Japan]) at a flow rate of 1.0 ml/min with the solvent system 0.1 M $NH_4H_2PO_4$ (pH 4.8)–methanol (4:1, vol/vol). The retention times for mandelic acid, mandelamide, mandelonitrile, and benzaldehyde were 2.2, 3.8, 17, and 24 min, respectively. The A_{254} was measured.

The optical purity of the mandelic acid was determined by

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direct analysis of the enantiomers. The reaction solution was adjusted to pH 8.5 with 1 N NaOH, after which unreacted mandelonitrile or mandelamide was extracted with dichloromethane. After the pH of the water layer had been adjusted to 7.0 with 1 N HCl, the solution was assayed by high-pressure liquid chromatography in a TSKgel Enantio L1 column (Tosoh Co., Tokyo, Japan) at a flow rate of 0.8 ml/min and with a solvent of 1 mM $CuSO_4$. The retention times of S-(+)- and R-(−)-mandelic acid were 9.5 and 10.5 min, respectively. The A_{254} was measured.

Partial purification of enzymes that hydrolyze mandelonitrile or mandelamide. All purification procedures were done at 0 to 5°C. The buffer solution used throughout was 0.03 to 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. Washed cells from 500 ml of culture broth were suspended in 50 ml of 0.03 M buffer and then disrupted by sonication in a Sonic 300 Dismembrator (Artex Systems Co.) run at maximum amplitude for 7 min. Cell debris was removed by centrifugation at 12,000 × g for 15 min, after which the supernatant was dialyzed against the same buffer. The solution obtained was applied to a DEAE-cellulose column (2.5 by 37 cm) equilibrated with 0.05 M buffer. After the column had been washed with the same buffer (300 ml), elution was done with a linear gradient of NaCl in the same buffer (0 to 0.5 M, 1,000 ml). The enzymes were eluted at 0.20 to 0.25 M NaCl in the same buffer. The active fractions (86 ml) were combined and concentrated to 10 ml in a Diaflo Cell 8050 (Amicon Co.) with a YM10 membrane. Saturated ammonium sulfate solution in 0.05 M buffer was added to a concentration of 30%. After the mixture had been stirred for 1 h, the precipitate was collected by centrifugation at 15,000 × g for 30 min. Saturated ammonium sulfate was added to the supernatant to 40% concentration. After this mixture had been stirred for 1 h, the precipitate formed was removed by centrifugation at 15,000 × g for 30 min and discarded. The ammonium sulfate concentration was increased to 60% saturation, and the mixture was again stirred for 1 h. The resulting precipitate was collected by centrifugation at 15,000 × g for 30 min. The 0 to 30% and 40 to 60% $(NH_4)_2SO_4$ precipitates were dissolved in 0.05 M buffer and then dialyzed against the same buffer.

Enzyme assay. Mandelonitrile-hydrolyzing activity was assayed in a reaction mixture (1 ml) containing 7.8 μmol of mandelonitrile, 100 μmol of Tris-HCl buffer (pH 8.5), and an appropriate amount of the enzyme. The reaction was performed at 30°C for 30 min and then stopped by adding 0.2 ml of 80% acetic acid to the reaction mixture. The amount of mandelic acid or mandelamide formed was measured by high-pressure liquid chromatography as described above. Mandelamide-hydrolyzing activity was also determined by measuring the amount of mandelic acid formed in this reaction, 7.8 μmol of mandelamide being used as the substrate. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of mandelic acid per min in the reaction mixture. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as the standard.

RESULTS

Screening of microorganisms. The ability to produce R-(−)-mandelic acid from mandelonitrile or mandelamide was examined in 10 culture strains that had been isolated during screening for the production of S-(+)-arylpropionic acids from their corresponding nitriles or amides (19) and in 482 type culture strains obtained from the American Type Culture Collection, Rockville, Md., and the Institute for Fermentation, Osaka, Japan. Thirty-eight distinct strains had mandelic acid-producing activity, representative strains of which are shown in Table 1. The optical purities of the products varied with the microorganism and substrate used. Of the active microorganisms, *A. faecalis* ATCC 8750 had both the highest activity and enantioselectivity in the reaction to produce R-(−)-mandelic acid from racemic mandelonitrile.

TABLE 1. Production of R-(−)-mandelic acid from mandelonitrile or mandelamide by selected bacteria and the optical purity of the product

Strain	Production[a] of R-(−)-mandelic acid from:	
	Mandelonitrile	Mandelamide
Corynebacterium nitrilophilus ATCC 21419	0.008 (18)	0.009 (17)
Alcaligenes faecalis ATCC 8750	0.291 (99)	0.136 (27)
Pseudomonas vesicularis ATCC 11426	ND[b]	0.036 (99)
Candida guilliermondii IFO 0454	ND	0.027 (99)
Rhodococcus sp. strain AK32	0.091 (15)	0.130 (30)
Corynebacterium sp. strain C5	0.118 (14)	0.200 (24)
Mycobacterium sp. strain AC777	ND	0.035 (21)
Acinetobacter sp. strain AK226	0.018 (83)	ND

[a] Micromoles of R-(−)-mandelic acid produced per milligram of dried cells per hour. Numbers in parentheses indicate the optical purity {[R/(S + R)] × 100} of the acid produced.
[b] ND, not detected (<0.001 μmol/mg/h).

Effect of carbon sources on growth and enzyme activity of *A. faecalis* ATCC 8750. Because *A. faecalis* ATCC 8750 does not use typical sugars such as glucose and sucrose, various carbon sources were tested for their effects on growth and enzyme activity in rich medium without glucose. Ammonium acetate and L-glutamic acid gave the best growth and the highest enzyme activity of the carbon sources tested (Table 2). Enzyme activity was induced when ammonium acetate or ammonium citrate was the carbon source, but

TABLE 2. Effects of various carbon sources on the growth and R-(−)-mandelic acid-producing activity of *A. faecalis* ATCC 8750[a]

Carbon source	Growth (OD_{610})	Activity (U)[b]	Optical purity (% enantiomeric excess)
None	2.9	0.0013	99
Glucose	2.9	0.0014	99
Acetonitrile	2.7	0.0027	99
Lactic acid	4.9	0.00005	99
Acetic acid	6.0	0.0013	99
Ammonium acetate	7.5	0.0138	99
Propionic acid	5.6	0.0027	99
Sodium citrate	6.3	0.0006	99
Ammonium citrate	7.4	0.0093	100
Fumaric acid	5.2	0.0004	99
L-Glutamic acid	9.7	0.0128	99
L-Aspartic acid	9.5	0.0064	100

[a] *A. faecalis* ATCC 8750 was grown at 32°C in a rich medium containing various carbon sources, but not glucose. Growth (the optical density at 610 nm [OD_{610}]) was measured after 20 h of incubation. After collection of the cells, the reaction with mandelonitrile was performed under screening conditions, and the optical purity of the R-(−)-mandelic acid produced was analyzed as described in Materials and Methods.
[b] One unit is the number of micromoles of R-(−)-mandelic acid produced per milligram of dried cells per minute.

TABLE 3. Effect of various inducers on the growth and *R*-(−)-mandelic acid-producing activity of *A. faecalis* ATCC 8750[a]

Inducer (%)	Growth (OD_{610})	Activity (U)	Optical purity (% enantiomeric excess)
None	8.7	0.000075	99
Acetonitrile (0.1)	8.7	0.000058	99
Propionitrile (0.1)	9.0	0.0022	99
n-Butyronitrile			
(0.1)	9.5	0.0450	100
(0.2)	10.6	0.0541	99
(0.3)	13.3	0.0593	100
n-Butyramide (0.1)	8.7	0.000075	99
n-Butyric acid (0.1)	8.4	0.00036	99
Isobutyronitrile (0.1)	8.7	0.0149	99
Benzyl cyanide (0.1)	8.4	0.0055	99
Mandelonitrile (0.1)	0.43	ND[b]	
Mandelamide (0.1)	8.9	0.00050	99
Mandelic acid (0.1)	8.3	0.00012	99
ε-Caprolactam			
(0.1)	9.1	0.0060	99
(0.2)	8.7	0.0147	100
(0.3)	8.8	0.0215	99
n-Butyronitrile (0.3) + ε-caprolactam (0.3)	10.3	0.0449	99
$LaCl_2$ (0.1 mM)	9.3	0.00024	100

[a] *A. faecalis* ATCC 8750 was grown at 32°C in a rich medium containing 1% ammonium acetate as the carbon source and various inducers (but not glucose and isobutyronitrile). Growth, activity, and optical purity were analyzed as discussed in footnotes *a* and *b* of Table 2.
[b] ND, not detected (<0.000002 U).

when ammonium ions were added together with ammonium acetate or L-glutamic acid as the carbon source, the enzyme activity induced was never higher than the activity in medium with only these carbon sources (data not shown). There was no difference in the optical purity of the *R*-(−)-mandelic acid produced by cells grown with various carbon sources.

Effects of various inducers on growth and enzyme activity. Enzyme activity was induced when *A. faecalis* ATCC 8750 was cultured with isobutyronitrile. The effects of various inducers on growth and enzyme activity were tested in the rich medium with ammonium acetate as the carbon source. Enzyme activity was induced by many compounds, including the aliphatic nitriles and ε-caprolactam (Table 3). $LaCl_2$, which is an effective substance for the expression of *Acinetobacter* sp. strain AK226 nitrilase (unpublished data), was not an effective inducer. The optical purity of the *R*-(−)-mandelic acid was not affected by the inducer used. *n*-Butyronitrile was the most efficient inducer, but it did not have a synergistic effect with ε-caprolactam. We therefore selected rich medium with 0.3% (wt/vol) *n*-butyronitrile as the best inducer.

Identification of *R*-(−)-mandelic acid. The *R*-(−)-mandelic acid formed from mandelonitrile by resting cells of *A. faecalis* ATCC 8750 was isolated and crystallized as described in Materials and Methods. Its nuclear magnetic resonance and infrared spectra and melting point were identical with those of an authentic sample. The specific rotation ($[\alpha]_D^{25}$) was −155° (c = 1, H_2O). A comparison of this value with the previously reported value (−155°) (8) shows that the optical purity of the *R*-(−)-mandelic acid formed was in a 100% enantiomeric excess, which agrees well with the results of the high-pressure liquid chromatography analysis.

Enzymes responsible for production of mandelic acid. Because *A. faecalis* ATCC 8750 hydrolyzed both mandelonitrile and mandelamide (Table 1), we partially purified the enzymes responsible for the production of mandelic acid and studied their characteristics. The purifications are summarized in Table 4. The activity that hydrolyzes mandelonitrile to produce *R*-(−)-mandelic acid (>99% enantiomeric excess) was detected, but no mandelamide-producing activity was found in any of the enzyme solutions obtained from the purification steps. We demonstrated that this microorganism has an enantioselective nitrilase but not a nitrile hydratase. In contrast, the activity of the mandelamide-hydrolyzing enzyme, the amidase, was lower than that of the mandelonitrile-hydrolyzing enzyme. The enantioselectivity of this amidase was not high because of the optical purity [$R/(S + R)$ = 24%] of the mandelic acid produced from mandelamide by the amidase; therefore, *A. faecalis* ATCC 8750 has an enantioselective nitrilase and a small amount of amidase.

Time course of *R*-(−)-mandelic acid-producing reaction. To determine the mechanism that functions in the production of *R*-(−)-mandelic acid from racemic mandelonitrile, we studied the time course of the reaction. Because mandelonitrile, a cyanohydrin, is in equilibrium in aqueous solution (mandelonitrile⇄benzaldehyde + HCN), the substrate amount 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. 1A), whereas no mandelamide was detected at any time during the reaction, evidence that *A. faecalis* ATCC 8750 has only the nitrilase for mandelonitrile. The yield of *R*-(−)-mandelic acid against the racemic substrate was 91%. The reason for this may be that residual *S*-mandelonitrile which is unreactive to the nitrilase is spontaneously racemized because of the chemical equilibrium (*S*-mandelonitrile⇄benzaldehyde + HCN⇄*RS*-mandelonitrile) and then used as the substrate for the nitrilase, thereby being consumed and converted to *R*-(−)-mandelic acid. The production of *R*-(−)-mandelic acid from benzaldehyde and HCN was studied to

TABLE 4. Summary of the partial purification of the enzymes acting in the production of mandelic acid[a]

Step	Protein (mg)	Mandelonitrile-hydrolyzing activity (I)		Mandelamide-hydrolyzing activity (II)		Ratio of two activities (I/II)
		U	U/mg	U	U/mg	
Cell extract	724	31.8	0.0439	0.968	0.00134	97.0/3.0
DEAE-cellulose	76.1	20.3	0.267	0.878	0.0115	95.9/4.1
$(NH_4)_2SO_4$ fractionation						
0–30%	8.16	9.96	1.22	ND[b]	ND	100/ND
40–60%	23.7	ND	ND	0.635	0.0268	ND/100

[a] Enzyme activity was determined as described in Materials and Methods.
[b] ND, not detected (<0.0001 U).

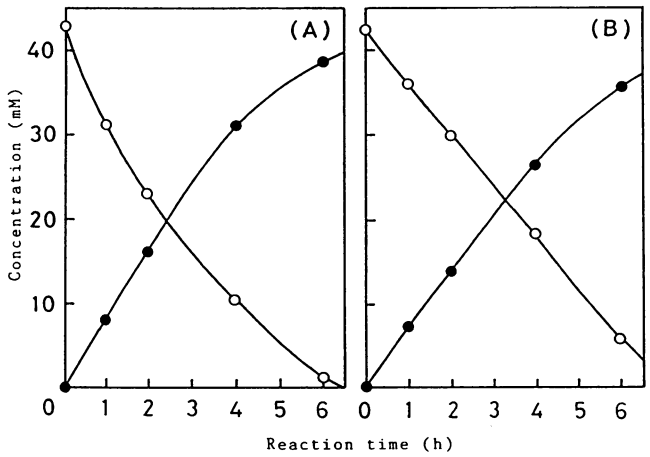

FIG. 1. Time course of R-(−)-mandelic acid production from mandelonitrile (A) and from benzaldehyde plus HCN (B). The reaction mixture (1 ml) containing 42 μmol of mandelonitrile or benzaldehyde and HCN (pH adjusted to 8.0), 100 μmol of potassium phosphate buffer (pH 8.0), and 3.2 mg (dry weight) of cells for mandelonitrile or 4.5 mg for benzaldehyde plus HCN was incubated in a shake flask at 32°C for 0, 1, 2, 4, and 6 h, after which the metabolites were analyzed as described in Materials and Methods. Symbols: ●, total amount of mandelonitrile and benzaldehyde; ○, amount of R-(−)-mandelic acid.

establish the mechanism. R-(−)-Mandelic acid was produced in the same way when benzaldehyde plus HCN was used as the substrate (Fig. 1B).

DISCUSSION

In general, enzymatic hydrolysis of nitrile compounds to the corresponding acids and ammonia takes place by two different pathways, by nitrilase and by a combination of nitrile hydratase and amidase. *A. faecalis* ATCC 8750 was shown here to have an enantioselective nitrilase for mandelonitrile and an amidase for mandelamide based on the partial purification of these enzymes. Moreover, we showed that only the R-enantioselective nitrilase is responsible for R-(−)-mandelic acid production from mandelonitrile by investigating the time course of this high-yield reaction. *A. faecalis* ATCC 8750 under the culture conditions reported here does not contain a nitrile hydratase for mandelonitrile. We did not, however, investigate whether this microorganism has a nitrile hydratase for other substrates.

Good growth with high enzyme activity was obtained with ammonium acetate or L-glutamic acid as the carbon source. Ammonium acetate appears to be an effective carbon source for the expression of the *A. faecalis* ATCC 8750 nitrilase, as it is for the nitrilase in *Acinetobacter* sp. strain AK226 (18, 19), but unlike with *Acinetobacter* sp. strain AK226, an inducer is essential for the induction of this nitrilase as it is for the nitrilase in *Alcaligenes faecalis* JM3 (12). In cells grown on medium with 0.3% *n*-butyronitrile, the nitrilase was expressed at 790-fold the value for cells grown on medium without the inducer.

Nitrilases from two *Nocardia* species (*rhodochrous* group) (5, 6), *Fusarium solani* (7), *Fusarium oxysporum* f. sp. *melonis* (4), *Arthrobacter* sp. strain J-1 (1), *Klebsiella pneumoniae* subsp. *ozaenae* (13), and *Rhodococcus rhodochrous* J-1 (9) preferentially hydrolyze aromatic nitriles such as benzonitrile or bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) but not aliphatic nitriles. Nitrilases that have broad substrate specificity and that hydrolyze both aromatic and aliphatic nitriles have been purified from *Acinetobacter* sp. strain AK226 (18) and *Rhodococcus rhodochrous* K22 (10). *A. faecalis* ATCC 8750 hydrolyzes mandelonitrile, an aliphatic nitrile with an aromatic ring. As this organism efficiently hydrolyzed such aliphatic nitriles as chloroacetonitrile but had difficulty with such aromatic nitriles as benzonitrile (data not shown), it might have a type of nitrilase that differs from the listed enzymes. The purification and characterization of the enzyme in this strain are now in progress.

The nitrilase in *A. faecalis* ATCC 8750 preferentially hydrolyzes R-mandelonitrile to produce R-(−)-mandelic acid. In the time course of R-(−)-mandelic acid production from racemic mandelonitrile however, no S-mandelonitrile remained, and the reaction yield from the racemate was 91%, evidence that the S-mandelonitrile remaining in the reaction is spontaneously racemized because of the chemical equilibrium and is then used as the substrate. Consequently, almost all was consumed and converted to R-(−)-mandelic acid (Fig. 2). A high reaction yield was also obtained for R-(−)-mandelic acid production from benzaldehyde plus HCN, indicating that the benzaldehyde and HCN are converted to RS-mandelonitrile because of chemical equilibrium and then used as the substrate, the reaction proceeding as described above.

In the enantioselective hydrolysis of racemates with esterase or lipase, unreacted enantiomer generally remains, and racemization is essential to recycle it as the substrate. To the best of our knowledge, we are the first to propose a new process in which an optically active α-hydroxy acid [such as R-(−)-mandelic acid] is produced from cyanohydrin or from an aldehyde and HCN with spontaneous racemization and a high reaction yield. Because many α-hydroxy acids of commercial importance are synthesized chemically from the corresponding nitriles or aldehyde compounds and HCN, our enzymatic process should make possible the

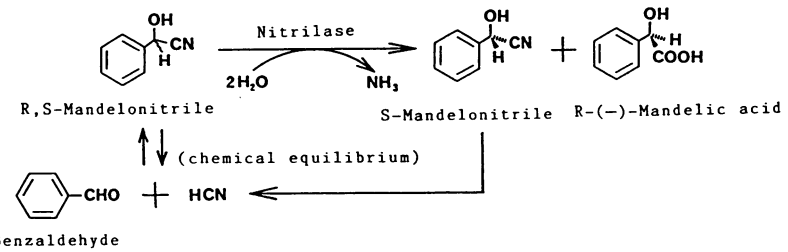

FIG. 2. Reaction mechanism for R-(−)-mandelic acid production from mandelonitrile or from benzaldehyde and HCN by *A. faecalis* ATCC 8750.

industrial production of a large variety of optically active α-hydroxy acids.

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