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Preparation of (*S*)-mandelic acids by enantioselective degradation of racemates with a new isolate *Pseudomonas putida* ECU1009

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Abstract—An enantioselective (*R*)-mandelate degrading bacterium, *Pseudomonas putida* ECU1009, was newly isolated from soil. The degradation activity of the bacterial cells was significantly enhanced by supplementing an optimal amount of racemic mandelic acid (0.4%), benzoylformic acid (0.4%), or benzoic acid (0.2%) to the culture medium as the enzyme inducer. Using the resting cells as a biocatalyst, three kinds of (*S*)-mandelic acids **1**–**3** were prepared with high isolated yields and enantiomeric excesses. Moreover, in a one-pot fermentation–transformation process using 1.25% (*RS*)-mandelic acid as the sole carbon/energy source for cultivation of the bacterium, (*S*)-mandelic acid **1** was accumulated after 48 h of bioconversion with 46.5% yield and >99% ee.
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1. Introduction

Mandelic acid and its derivatives are a class of chiral synthons for the production of various pharmaceuticals, such as semi-synthetic penicillins, cephalosporins, and antiobesity agents.[1–3] Many methods have been reported for the preparation of enantiomerically pure (*S*)- or (*R*)-mandelic acid (**1**).[4–10] For example, (*S*)-**1** has been separated from racemic mixtures by means of high-pressure crystallization.[11] It has also been prepared by the enzyme-catalyzed synthesis of (*S*)-cyanohydrins and subsequent hydrolysis into (*S*)-α-hydroxy-carboxylic acids,[12] from benzoylformic acid by fermentation with *Lactobacillus* genus (e.g., *Lactobacillus bulgaricus* and *Lactobacillus plantarum*) or *Pediococcus* genus (e.g., *Pediococcus parvulus* and *Pediococcus pentosaceus*),[13] or from methyl mandelate by lipase-catalyzed hydrolysis.[14] However, little is known about the biocatalytic conversion of (±)-**1** into the (*S*)-enantiomer,[9] in spite of many reports regarding the bioproduction of (*R*)-mandelate.[4,8–10] This prompted us to screen for microorganisms to produce (*S*)-**1** from the racemic mixture.

The enzymes involved in the mandelate metabolic pathway allow various *Pseudomonas* species to utilize either one or both of the mandelate enantiomers as its sole carbon source.[15–18] Usually, the catabolism of the (*R*)-mandelate requires the presence of mandelate racemase to equilibrate the enantiomers and generate (*S*)-mandelate, which can then be oxidatively degraded to benzoate by the remaining enzymes in the pathway. Though *Aspergillus sydowi* IFO 4284 and *Fusarium oxysporum* IFO 5942 have previously been reported as being capable of selectively oxidizing (*R*)-**1** from its racemate, the activity of these two fungal strains were rather low.[9] Herein, we report a newly isolated bacterial strain, *Pseudomonas putida* ECU1009, which is capable of degrading the (*R*)-enantiomer of racemic mandelate and affording (*S*)-mandelate in high enantiomeric excess (Scheme 1). This method gives a theoretical yield of 50%, as do other classical kinetic resolutions. However, the method possesses several advantages over the other processes mentioned above: (*R*)-**1** and its intermediates can be completely degraded, therefore only a simple extraction step is needed to gain the final product in fairly high purity. Hence, the product isolation and purification is

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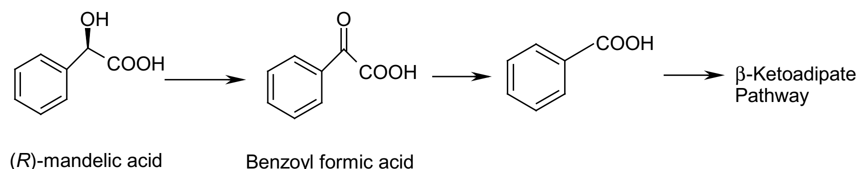


Scheme 1. Proposed mandelate pathway of *Pseudomonas putida* ECU1009.

simple and efficient. Although the theoretical yield of (*S*)-**1** is limited to 50%, an enantioselective two-step stereoinversion[8] of (±)-**1** to (*S*)-**1** can be considered to overcome this disadvantage.

2. Results and discussion

2.1. Screening of microorganisms

Bacteria with mandelate-degrading activities were isolated from soil samples through a two-step enrichment cultivation procedure on a minimal salt medium (MSM) containing benzoylformic acid (0.2%) as the sole carbon source with the first round of screening for benzoylformic acid-degrading activity, and the second round of screening for selective degrading activity on (*S*)- or (*R*)-**1**. In the first round, benzoylformic acid-degrading strains were isolated after being grown at 30 °C on MSM for 3 days; about 140 bacterial strains among 200 isolates were considered to be significantly active on benzoylformic acid. In the second round of screening, about 10 strains gave >98% enantiomeric excess of mandelic acid, of which nine strains could selectively degrade (*S*)-**1**. Finally, a strain marked as ECU1009 was chosen for further study due to its high activity and enantioselectivity toward (*R*)-**1**. This strain was later identified to be *P. putida* by the Shanghai Center for Disease Control based on its taxonomical characteristics (data not shown) and thus designated as *P. putida* ECU1009. To the best of our knowledge, this is the first example of the enantioselective degradation of (*R*)-**1** from racemate by the redox system of *P. putida*.

2.2. Enantioselective degradation of mandelic acid derivatives

The enantioselective degradation of (±)-**1** was first examined using the resting cells of *P. putida* ECU1009 as the biocatalyst. The molecular structures of two key intermediates, benzoic acid and benzoylformic acid, were confirmed by mass spectra and NMR (see Sections 4.4.1 and 4.4.2). The time-dependant changes of the substrate and product concentrations are shown in Figure 1. Compound (*S*)-**1** was only slightly degraded after 34 h while the residual mandelic acid concentration was 29.5 mM (analytical yield 49%; ee 99%) and the benzoylformic acid concentration was very low during the whole process of biotransformation. A possible intermediate of benzaldehyde was not detected in our case. The concentration of the accumulated benzoate as an intermediate of (*R*)-**1** reached the maximum at 18 h and it was further degraded completely after 44 h.

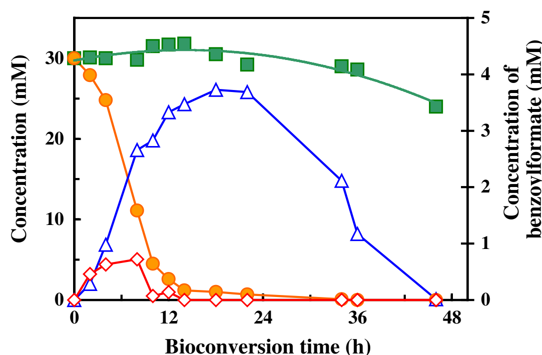


Figure 1. Time course of enantioselective degradation of (±)-mandelic acid by resting cells of *Pseudomonas putida* ECU1009. The reaction was performed at 30 °C and 160 rpm in 80 mL of 100 mM potassium phosphate buffer (pH 6.0) containing 60 mM of (±)-**1** with 8.47 g DCW/L of resting cells. Symbols: (■) (*S*)-**1**; (●) (*R*)-**1**; (◇) benzoylformate; (△) benzoate.

An average enantiomeric ratio (*E*-value) of about 130 ± 30 was obtained according to the equation of Chen et al.:[19] $E = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$.

Poor growth and a lower degrading activity of cells were observed if aged cells were used. In order to overcome these drawbacks, the strain was grown freshly on a slant medium (SM) for 24–48 h at 30 °C each time before shake-flask cultivation. The optimal temperature and pH of the enantioselective degradation were 30 °C and 6.0, respectively. The degrading activity of (*R*)-**1** was significantly enhanced when an appropriate amount of racemic mandelic acid, benzoylformic acid, or benzoic acid was added to the culture medium (Fig. 2). The optimal concentrations of these inducers were 0.4% for racemic mandelic acid, 0.4% for benzoylformic acid and 0.2% for benzoic acid. At 0.4% of benzoic acid, poor growth and a lower degrading activity of cells were observed, indicating that the benzoic acid as inducer was more toxic to the cells than racemic mandelic acid or benzoylformic acid. At 60 mM of the racemic mandelate (±)-**1**, the bacterial cells exhibited the maximum degrading activity on (*R*)-**1**. Under the optimal reaction conditions, (*S*)-**1** was recovered in 42% isolated yield and 99% ee after 24 h of biotransformation. The substrate spectrum of the newly discovered biocatalyst was examined using several derivatives of mandelic acid, as shown in Table 1. *P. putida* ECU1009 also showed high enantioselective activities on (*R*)-mandelate derivatives with substitution groups (–OH or –Cl) at the *para*-position of the aromatic ring, for example, **2** and **3**. However, when a substrate with a substitution at the *meta*- or *ortho*-position, for example, **4** and **5**, either the enzy-

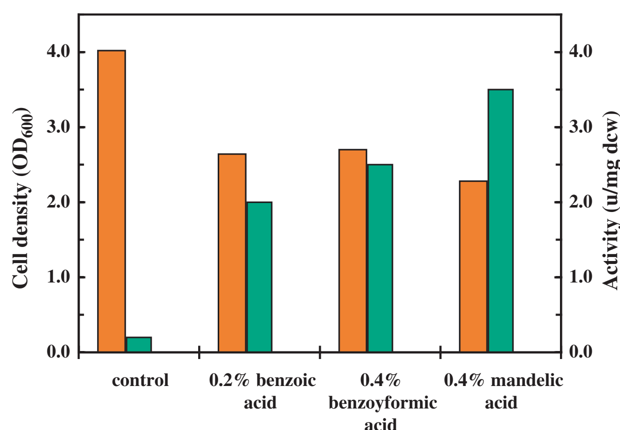


Figure 2. Effect of (±)-mandelic acid, benzoylformic acid and benzoic acid on cell growth and production of (*R*)-mandelate-degrading enzyme by *Pseudomonas putida* ECU1009. One unit (u) of activity was defined as the amount of enzyme needed for the degradation of 1 μg (*R*)-**1** per minute at 30 °C and pH 6.0. Assay conditions: (±)-**1**, 60 mM, 20 mL; incubation for 2 h. Orange bar: cell density reflected by optical density at 600 nm; Green bar: enzyme activity (units per milligram of dry cell weight).

matic activity or the enantioselectivity was significantly decreased, although the reasons are as yet unclear.

Furthermore, *P. putida* ECU1009 was able to grow with (*R*)-**1** of the racemate (1.25%) as the sole carbon/energy source, leaving (*S*)-**1** in 46.5% analytical yield and >99% ee after 48 h of cultivation/transformation on an optimized fermentation medium. As (±)-**1** is produced commercially on a large scale at a very low cost by chemical synthesis, its use as a raw material for (*S*)-**1** production would be beneficial for simplifying the post-reaction separation of the (*S*)-isomer from the racemic mixture.

3. Conclusions

Using racemic mandelic acids as starting materials, three kinds of (*S*)-mandelic acids **1**–**3** were efficiently prepared with high isolated yields and ee. The degrading activity of (*R*)-mandelic acids can be significantly enhanced by supplementing 0.4% racemic mandelic acid, 0.4% benzoylformic acid or 0.2% benzoic acid to the culture medium. Moreover, using (±)-**1** as the sole carbon source, (*S*)-**1** could also be obtained after 48 h of fermentation with 46.5% analytical yield and >99% ee.

4. Experimental

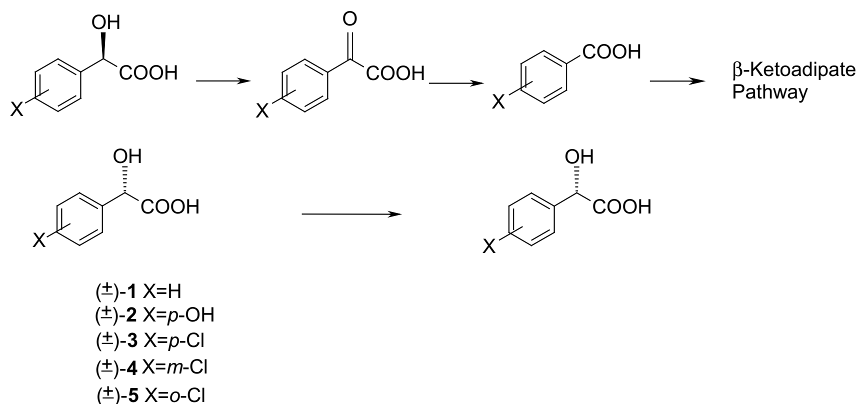
4.1. General

Minimal salt medium (MSM): 0.2% benzoylformic acid, 0.2% $(NH_4)SO_4$, 0.1% yeast extract, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl, 0.05% $MgSO_4$, 1.8% agar, pH 7.0.

Medium for slant culture (SM): 1.5% glycerol, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl, 0.05% $MgSO_4$, 0.4% racemic mandelic acid, 1.8% agar, pH 7.0.

Fermentation medium (FM): the same as SM but without agar and mandelic acid.

Table 1. Preparation of (*S*)-mandelic acids by enantioselective degradation of racemates using resting cells of *Pseudomonas putida* ECU1009[a]



Entry	Substrate	Concn (mM)	Time (h)	Yield[b] (%)	ee (%)[c]
1	(±)-**1**	60	24	42	>99
2	(±)-**2**	60	24	28	>99
3	(±)-**3**	60	48	47	>99
4	(±)-**3**	40	36	35	>99
5	(±)-**4**	40	36	56	43
6	(±)-**4**	40	48	45	85
7	(±)-**5**	40	48	90	3

[a] The reactions were carried out with 14.0 g DCW/L of resting cells in 100 mM potassium phosphate buffer (pH 6.0) at 30 °C, and 160 rpm.
[b] Isolated yield after purification by silica gel column chromatography.
[c] ee (%) were determined by chiral HPLC.

Benzoylformic acid and racemic mandelic acids (±)-**1**–**5** were purchased from Guangde Chemicals Co. Ltd, China. All other chemicals were also from commercial sources and of reagent grade.

4.2. Microorganism, growth and biotransformation conditions

P. putida ECU1009 was maintained on SM at 30 °C. The strain, after being grown on SM for 24–48 h, was first pre-cultivated in 5 mL FM for 12 h, then inoculated into 500 mL Erlenmeyer flasks containing 95 mL of the FM and shaken at 30 °C and 160 rpm. After 12 h, appropriate inducers were added to the FM. After 24 h of cultivation, cells were harvested from the culture broth, washed and re-suspended in 100 mM potassium phosphate buffer (pH 6.0) containing 60 mM (±)-**1**.

4.3. Bioconversion process by resting cells

The bioconversion process was monitored with HPLC by withdrawing 0.6 mL of samples at fixed time intervals. The cells were removed by centrifugation and 0.1 mL of the supernatant was used for the determination of the product and substrate concentrations by HPLC (Shim-pack VP-ODS, Shimadzu Co., Japan) with an elution system of 10 mM KH_2PO_4–CH_3OH (87:13, v/v) at a flow rate of 0.8 mL/min and the UV absorbance at 225 nm. The remaining 0.5 mL sample was used for HPLC determination of the enantiomeric excess (ee) of mandelic acid after derivation into methyl mandelate with methanol and trimethylchlorosilane, using a chiral column (Chiralcel OD, Daicel Co., Japan) eluted with hexane–isopropanol (90:10, v/v, 0.8 mL/min) and detected at 228 nm.

4.4. Preparation of (*S*)-1, its intermediates and derivatives by resting cells

P. putida ECU1009, was grown on SM for 24–48 h at 30 °C, pre-cultivated in 5 mL liquid medium (FM) for 12 h, inoculated into 500 mL Erlenmeyer flasks containing 95 mL of FM and shaken at 30 °C and 160 rpm. After 12 h, 0.2% benzoic acid was added to the culture medium. After 24 h of cultivation, cells harvested from 250 mL of culture broth were re-suspended in 50 mL of 100 mM potassium phosphate buffer (pH 6.0) containing a certain concentration of (±)-**1**, (±)-**2**, (±)-**3**, (±)-**4**, or (±)-**5**. After a certain time of bioconversion, the cells were removed by centrifugation and the supernatant acidified with concentrated H_2SO_4 to pH 1.0 and extracted with ethyl acetate. The ethyl acetate layer was collected, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain a crude crystal of (*S*)-(+)-mandelic acids. The acids were purified by silica gel column chromatography. The eluent components are toluene–ethyl acetate–formic acid (5:1:0.2, v/v/v) for **3**, **4**, **5**; benzene–ethyl acetate–formic acid (5:1:0.5, v/v/v) for **1**, benzoylformic acid and benzoic acid; and benzene–methanol–formic acid (5:1:0.5, v/v/v) for **2**.

4.4.1. Benzoylformic acid. White powder. Yield 2% (isolated at 6 h). ^{1}H NMR (500 MHz, D_2O): δ/ppm: 7.377 (m, 2H), 7.518 (s, 1H), 7.860 (m, 2H). MS (ESI) *m*/*z*: 150 (M^+); $C_8H_6O_3$ requires 150.

4.4.2. Benzoic acid. White powder. Yield 80% (isolated at 18 h). ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm: 7.540 (m, 3H), 8.170 (m, 2H); MS (ESI) *m*/*z*: 122 (M^-); $C_7H_6O_2$ requires 122.

4.4.3. (*S*)-Mandelic acid (*S*)-1. White powder. Yield 42%. $[\alpha]_D^{25} = +152.0$ (*c* 1.0, H_2O), >99% ee. HPLC conditions for its methyl ester: Chiralcel OD, hexane–2-propanol (90:10, v/v; 0.8 mL/min), detector 228 nm; (*S*) 18.7 min, (*R*) 25.7 min. ^{1}H NMR (500 MHz, D_2O): δ/ppm: 5.260 (s, 1H), 7.350 (m, 5H).

4.4.4. (*S*)-*p*-Hydroxyl-mandelic acid (*S*)-2. White powder. Yield 28%. $[\alpha]_D^{25} = +140.0$ (*c* 1.1, H_2O), >99% ee. HPLC conditions for its methyl ester: Chiralcel OD, hexane–2-propanol (88:12, v/v; 0.8 mL/min), detector 228 nm; (*S*) 18.7 min, (*R*) 25.7 min. ^{1}H NMR (500 MHz, D_2O): δ/ppm: 5.200 (s, 1H), 6.770 (m, 2H), 7.254 (m, 2H).

4.4.5. (*S*)-*p*-Chloro-mandelic acid (*S*)-3. White powder. Yield 47%. $[\alpha]_D^{25} = +128.6$ (*c* 1.0, H_2O), >99% ee. HPLC conditions for its methyl ester: Chiralcel OD, hexane–2-propanol (98:2, 0.8 mL/min), detector 228 nm; (*S*) 19.0 min, (*R*) 22.0 min. ^{1}H NMR (500 MHz, D_2O): δ/ppm: 5.150 (s, 1H), 7.350 (m, 4H).

4.4.6. (*S*)-*m*-Chloro-mandelic acid (*S*)-4. White powder. Yield 45%. $[\alpha]_D^{25} = +105.8$ (*c* 1.0, H_2O), 85% ee. HPLC conditions for its methyl ester: Chiralcel OD, hexane–2-propanol (98:2, 0.8 mL/min), detector 228 nm; (*S*) 18.3 min, (*R*) 21.3 min. ^{1}H NMR (500 MHz, D_2O): δ/ppm: 5.200 (s, 1H), 7.320 (m, 3H), 7.426 (m, 1H).

4.4.7. (*S*)-*o*-Chloro-mandelic acid (*S*)-5. White powder. Yield 90%. $[\alpha]_D^{25} = +4.0$ (*c* 1.0, H_2O), 3% ee. HPLC conditions for its methyl ester: Chiralcel OD, hexane–2-propanol (98:2, 0.8 mL/min), detection 228 nm; (*S*) 18.0 min, (*R*) 22.0 min. ^{1}H NMR (500 MHz, D_2O): δ/ppm: 5.560 (s, 1H), 7.284 (m, 2H), 7.350 (m, 1H), 7.406 (m, 1H).

4.5. Preparation of (*S*)-1 by fermentation

P. putida ECU1009, grown on SM for 24 h at 30 °C, was pre-cultivated in 10 mL FM for 12 h and inoculated into 500 mL Erlenmeyer flasks containing 100 mL of the liquid medium composed of 1.25% racemic mandelic acid, 0.1% NH_4NO_3, 0.6% K_2HPO_4, 0.3% KH_2PO_4, 0.1% NaCl, 0.05% $MgSO_4$, and 0.05% yeast extract at pH 7.0, 30 °C, and 160 rpm. After 48 h, the chemical yield and ee of mandelic acid were determined by HPLC as described above.

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