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Optical Resolution of DL-Pipecolic Acid by Fermentation Using *Pseudomonas* sp. PA09

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Abstract *Pseudomonas* sp. PA09 was isolated from farm soil and used for the optical resolution of D-pipecolic acid from DL-pipecolic acid. The strain PA09 consumed L-pipecolic acid preferentially as the sole carbon and energy source, thus accumulating D-pipecolic acid in the culture broth. Optimization to improve the enantiomeric excess and yield was performed. The time course experiment showed that the strain PA09 consumed L-pipecolic acid almost to completion after 25 h of cultivation, and the enantiomeric excess and the yield (% of residual D-pipecolic acid) were 99.8 and 96.0%, respectively.

Key words: Fermentation, D-pipecolic acid, optical resolution, *Pseudomonas* sp.

Pipecolic acid (2-piperidine carboxylic acid), an unnatural amino acid, it is known as an active regulator of synaptic transmission in mammals [20]. It is also a precursor or structural component in the synthesis of various drugs, such as swainsonine [8], thioridazine [4], L-N-N-propylpipecolic acid-2,6-xylidide (Ekenstam, 1987, U.S. Patent 4,656,576), FK 506 [7], demethoxyrapamycin [3], amphomycins [16], and piperidine alkaloids [15]. Recently, pipecolic acid has been shown to be a modifier of nonapeptide which is a protease inhibitor for human immunodeficiency virus [19]. D-pipecolic acid has often been found to be an active intermediate for the drugs mentioned above. Thus, to suppress any side effects due to the racemates used and to achieve the maximal efficiency rate, it is necessary to obtain optically pure D-pipecolic acid for use in the preparation of the drugs. In fact, it is a requirement of the US Food and Drugs Administration for pharmaceutical companies to prepare and test both enantiomers of any new chiral drug before being marketed [11].

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Several chemical methods [1, 17] have been developed to prepare D-pipecolic acid but, unfortunately, they are complicated or impractical. In general, it is often economical to employ microorganisms for selective degradation or transformation of a chemical, as shown in many previous studies [5, 6, 10, 13, 14, 18]. Mochizuki *et al.* [15] reported that *Alcaligenes* sp. degraded L-pipecolic acid selectively while leaving D-isomer. Christine *et al.* [4] have employed a kinetic resolution using lipase which catalyzed the transformation of (±)-octyl pipecolic acid to L-pipecolic acid. In this study, we report on the optical resolution of DL-pipecolic acid by fermentation with *Pseudomonas* sp., showing that, under an optimized condition, the isolated strain produced D-pipecolic acid with higher optical purity and yield than previously reported [1, 4, 15, 17].

Materials and Methods

Chemicals

D-, L-, and DL-pipecolic acid, D-amino acid oxidase, flavin adenine dinucleotide (FAD), and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of Analytical Reagent grade.

Strain Selection

A farm soil was collected and used to isolate strains that preferentially assimilate L-pipecolic acid from DL-pipecolic acid. The soil was suspended in a sterile 0.85% (w/v) NaCl solution and the suspension was filtered through a Whatman No. 2 filter paper (Whatman Lab., Clifton, NJ, U.S.A.). A minimal medium (pH 7.0) containing 0.1% DL-pipecolic acid, 0.3% K_2HPO_4, 0.3% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.001% $CaCl_2$, and 0.1% trace elements (pH 7.0) was inoculated with the filtrate. The trace elements were composed of 0.3 mg/l H_3BO_3, 0.2 mg/l $CuSO_4 \cdot 5H_2O$, 2.5 mg/l $FeCl_3 \cdot 6H_2O$, 0.2 mg/l

$MnCl_2 \cdot 4H_2O$, 0.75 mg/l $ZnCl_2$, 0.1 mg/l $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$, 0.15 mg/l $CoSO_4 \cdot 7H_2O$. The enrichment culture continued at 30°C with gyratory shaking at 200 rpm. After 24 h, 0.2 ml of the culture broth was diluted and smeared on a 2% agar plate containing the minimal medium. Each strain on the plates was picked and grown on the agar plate for 16 h. Isolated strains were then stored at 4°C until use.

Each strain was tested for the preferential assimilation of L-pipecolic acid as follows: Seed culture was prepared by adding a loopful of cells from an agar plate to a test tube containing 5 ml of Lennox medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.0) and grown at 30°C with shaking. After 24 h, a loopful of seed culture was added to the test tube containing 5 ml of the minimal medium. After 16 h cultivation at 30°C with gyratory shaking at 200 rpm, each culture broth was centrifuged and passed through 0.22 m syringe filters (Micron Separations, Westboro, MA, U.S.A.) to remove any particulate matter. Both the amounts of D-pipecolic acid and DL-pipecolic acid in the filtrate were measured as described below. We also measured the growth of the strains by optical density at 610 nm (OD_{610}), since it might affect both the enantiomeric excess and the cultivation time (i.e. the economy of the process).

Optical Resolution of DL-Pipecolic Acid by Cultivation

For the removal of L-pipecolic acid from the racemate by assimilation of the isolated strain, the isolated strain on the agar plate was first grown in 7 ml of the minimal medium at 30°C with shaking. After 9 h, 1 ml of the seed culture was added to a 500-ml Erlenmeyer flask containing 100 ml of the minimal medium. The culture was then grown with gyratory shaking at 130 rpm. As the isolated strain assimilated L-pipecolic acid, the enantiomeric excess (e.e.) (%) [=(D-L)/(D+L)×100] increased. The maximum theoretical yield by fermentation is 50%, since half of the racemate are assimilated by microorganisms. Thus, we considered the initial concentration of D-pipecolic acid as 100%. Other experimental conditions are described in the figure legend and tables.

Analysis of DL-Pipecolic Acid and D-Pipecolic Acid

The amount of DL-pipecolic acid in the filtrate was quantitatively measured according to a previous method [17]. In brief, 0.4 ml of the filtrate was mixed with 2.8 ml of acetic acid solution containing 0.15% (w/v) ninhydrin and incubated in boiling water for 35 min. It was then cooled to room temperature and the absorbance level was measured at 565 nm with a spectrophotometer (Novaspec II, LKB, Cambridge, England).

For quantitative analysis of D-pipecolic acid [12], 1 volume of the filtrate was mixed with 1 volume of the reaction mixture containing 0.2 U/ml of D-amino acid oxidase, 0.2 mol/ml of FAD, and 25 mM pyrophosphate (pH 8.3) (all at the final concentration). The mixture was incubated at 30°C for 1 h and 2 volumes of 12.5% (w/v) trichloroacetic acid (pH 5.0) was then added. The mixture was incubated again at 50°C for 30 min and then cooled to room temperature. Absorbance was measured at 320 nm. The amount of L-pipecolic acid was calculated by subtracting the amount of D-form from DL-form. All the values in the Tables are the averages of duplicates.

Characterization of Isolated Strain

The isolated strain was grown on the Luria-Bertani agar plate and subjected to the Gram test. Morphology of the isolated strain was examined with a transmittance electron microscope (ZEISS EM109, FGR) after staining with 2% (w/v) phosphotungstic acid (pH 6.8). The strain was also identified by morphological, biochemical, and cultural methods as described in Bergey's Manual of Systematic Bacteriology [9].

RESULTS AND DISCUSSION

Screening of Strains

Seven isolates were chosen according to the ability to grow on DL-pipecolic acid as the sole carbon and energy source (Table 1). After 16 h cultivation at 30°C, the optical density ranged from 0.43 to 0.93. Some of the strains (PA14, PA17, PA24, and PA25) assimilated both enantiomers equally well and others (PA09 and PA10) assimilated the L-form somewhat better than the D-form. Strains PA14, PA24, and PA25 grew faster than PA09 and PA10 which preferentially assimilated the L-form, indicating that balanced assimilation of both enantiomers might help the growth of the former strains. Strain PA33 assimilated the D-form better than the L-form, as indicated by the negative enantiomeric excess (e.e.) (Table 1). Strain PA09 had a similar growth as PA10, but had a higher e.e. (69.8%) than PA10 (48.8%).

Table 1. Seven isolated strains that were grown on DL-pipecolic acid as the sole energy and carbon source[a].

Strains No.	Growth (OD_{610})	Residual D-pipecolic acid[b] (g/l)	Enantiomeric excess (%)
PA09	0.59	0.454	69.8
PA10	0.52	0.460	48.8
PA14	0.77	0.368	8.8
PA17	0.55	0.381	19.4
PA24	0.71	0.178	31.0
PA25	0.93	0.140	29.4
PA33	0.43	0.284	-4.2

[a]Cultivations were carried out for 16 h at 30°C in the minimal medium (pH 7.0) (see Materials and Methods) containing 0.1% DL-pipecolic acid.
[b]The amount of D-pipecolic acid that was left as unmetabolized during the cultivation. The initial amount of D-pipecolic acid was 0.5 g/l.

Table 2. Physiological and biochemical characteristics of the strain PA09.

Characterizations	PA09
Morphological characteristics	
Mobility	+[a]
Shape	rod
Gram test	-
Assimilation of carbon compounds	
D-Glucose	+
Sucrose	+
Succinate	-
D-Fructose	-
Citrate	-
L-Sorbose	-
L-Rhamnose	-
D-Arabinose	-
Maltose	-
Citrate	-
Dulcitol	-
Physiological characteristics	
Oxidase test	+
Oxygen test	+
Oxygen requirement	aerobe
O/F test	oxidative
Reduction of nitrates to nitrites	-
Methyl red test	-
Starch hydrolysis	-
Gelatin hydrolysis	-
Indol test	-
Arginine dehydrolase	+

[a]+, positive; -, negative.

Therefore, the strain PA09 was chosen and used in the subsequent experiments. Strain PA09 had a rod shape and flagella, and was Gram negative. The bacterium was identified by the above findings, together with other tests shown in Table 2, as *Pseudomonas* sp.

Effect of Substrate Concentration on the Resolution of DL-Pipecolic Acid

The concentration of the racemate in the minimal media was varied from 1 to 4% to observe the effect of substrate concentration on the assimilation of L-pipecolic acid (Table 3). The growth of strain PA09 was somewhat suppressed when the concentration was 4%. The e.e. decreased from 99.8 to 8.4% with increasing concentration of DL-pipecolic acid. About 28% of the initial D-pipecolic acid was assimilated when the concentration was 1%, compared to ~5% of the initial D-pipecolic acid when the concentration was more than 1%. However, the residual amount of D-pipecolic acid increased as the initial concentration of DL-pipecolic acid increased (Table 3). Considering the growth, the residual amount of D-pipecolic acid, and the e.e., 3% of DL-pipecolic acid was used in the following experiments.

Table 3. Effect of substrate concentration on the resolution of DL-pipecolic acid by *Pseudomonas* sp. PA09[a].

DL-Pipecolic acid (%)	Growth (OD_{610})	Residual D-pipecolic acid[b] (g/l)	Enantiomeric excess (%)
1	4.60	3.60	99.8
2	4.49	9.60	56.2
3	4.41	14.6	49.0
4	3.77	19.2	8.4

[a]Cultivations were carried out for 24 h at 30°C in the minimal medium (pH 7.0) (see Materials and Methods) containing various concentrations of DL-pipecolic acid.
[b]The amount of D-pipecolic acid that was left as unmetabolized during the cultivation. The initial amount of D-pipecolic acid was 5.0, 10, 15, 20 g/l for 1, 2, 3, 4% of DL-pipecolic acid, respectively.

Effects of Inorganic and Organic Nitrogens on the Resolution

To investigate the effect of inorganic nitrogen on the resolution, while maintaining the concentration of the racemate at 3%, the concentration of various inorganic nitrogens, such as $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4NO_3, NH_4Cl, $(NH_4)_2CO$, and KNO_3, was adjusted so that the carbon/nitrogen (C/N) ratio was 40. The growth and e.e. was higher with NH_4NO_3 than with the other inorganic nitrogens (data not shown). Then, the concentration of NH_4NO_3 was varied so that the C/N ratio ranged from 10 to 60 (Table 4). The growth and e.e. decreased significantly with a C/N ratio higher or lower than 40. When the C/N ratio was 40, the e.e. was 50.6% and the residual D-pipecolic acid was 97.3% of the initial concentration.

By adding organic nitrogen to a minimal medium, it may expedite the growth of microbes. Therefore, various organic nitrogens were added at the concentration of 0.1% to investigate the effect on the resolution of the racemate by *Pseudomonas* sp. PA09, while maintaining the concentrations of the racemate and NH_4NO_3 at 3 and 0.12%, respectively. Table 5 shows the growth and the e.e. after 24 h cultivation.

Table 4. Effect of carbon/nitrogen ratio on the resolution of DL-pipecolic acid by *Pseudomonas* sp. PA09[a].

C/N Ratio	Growth (OD_{610})	Enantiomeric excess (%)
10	0.500	6.40
20	2.31	28.8
30	4.06	45.4
40	4.35	50.6
50	3.80	44.0
60	3.73	32.8

[a]Cultivations were carried out for 24 h at 30°C in the minimal medium (pH 7.0) (see Materials and Methods) containing 3% DL-pipecolic acid and various concentrations of NH_4NO_3 according to the indicated C/N ratio.

Table 5. Effect of organic nitrogens on the resolution of DL-pipecolic acid by *Pseudomonas* sp. PA09[a].

Nitrogen sources (0.1% w/v)	Growth (OD_{610})	Enantiomeric excess (%)
None	4.26	49.8
Yeast extract	5.47	63.0
Peptone	4.98	52.2
Casamino acid	4.73	34.4
Tryptone	4.51	33.4
Soytone	6.03	30.0

[a]Cultivations were carried out for 24 h at 30°C in the minimal medium (pH 7.0) (see Materials and Methods) containing 3% DL-pipecolic acid, 0.16% NH_4NO_3, and various kinds of organic nitrogens.

The results indicated that yeast extract gave the highest e.e. (63.0%) when compared with other organic nitrogens. The highest growth was obtained with soytone but the e.e. was about 30%. In the following experiments, the concentrations of the racemate, NH_4NO_3, and yeast extract were 3, 0.12, and 0.1%, respectively.

The Effects of pH and Temperature on the Optical Resolution

While the composition of the media and other conditions were kept constant, as indicated in Table 6, the pH level of the media was varied from 5.5 to 8.0. While there was no significant difference in the growth with different pHs, it is interesting to note that the highest e.e. (64.6%) was obtained at pH 6.5.

Strain PA09 was grown in the above-determined media, while the temperature varied from 27 to 39°C (Table 7). The growth increased with increasing temperature but the e.e. (85.6%) peaked at 33°C. With temperatures either lower or higher than 33°C, the residual D-pipecolic acid decreased. Thus, the culture condition of 33°C and pH 6.5 was employed in the following experiments.

Time Course of the Optical Resolution of DL-Pipecolic Acid by *Pseudomonas* sp. PA09

Figure 1 shows the time course of the enantioselective assimilation of L-pipecolic acid by strain PA09. The growth continued for 25 h where most of the L-pipecolic acid was exhausted, indicating that the strain used L-pipecolic acid preferentially as the sole energy and carbon source among the racemates. The specific growth rate (μ_{max}) was 0.160 h^{-1} and doubling time (t_d) was 4 h 20 min. However, the growth remained constant for a while after 25 h, and then it continued again. The second growth coincided with further decrease in the concentration of DL-pipecolic acid, indicating that strain PA09 began to assimilate D-pipecolic acid. At 25 h of cultivation, the e.e. was 99.8% and the residual D-pipecolic acid was 96.0% of the initial concentration. It is highly likely that L-pipecolic acid dehydrogenase [2] was first induced in the presence of both enantiomers and next was D-pipecolic acid dehydrogenase

Table 6. Effect of initial pH on the resolution of DL-pipecolic acid by *Pseudomonas* sp. PA-09.

Initial pH	Growth (OD_{610})[a]	Enantiomeric excess
5.5	5.61	47.8
6.0	5.52	52.4
6.5	5.30	64.6
7.0	5.43	62.2
7.5	5.56	53.6
8.0	5.87	46.4

[a]Cultivations were carried out for 24 h at 30°C in the minimal medium (see Materials and Methods) containing 3% DL-pipecolic acid, 0.12% NH_4NO_3, and 0.1% yeast extract. The pH of the medium was varied as indicated.

Table 7. Effect of temperature on the resolution of DL-pipecolic acid by *Pseudomonas* sp. PA09[a].

Temperature (°C)	Growth (OD_{610})	Residual D-pipecolic acid[b] (g/l)	Enantiomeric excess (%)
27	2.51	13.9	8.00
30	5.31	14.6	64.6
33	5.88	14.5	85.6
36	6.45	12.4	67.4
39	7.30	10.9	57.2

[a]Cultivations were carried out for 24 h in the minimal medium (pH 6.5) (see Materials and Methods) containing 3% DL-pipecolic acid, 0.12% NH_4NO_3, and 0.1% yeast extract. The temperature during the cultivation varied as indicated.
[b]The amount of D-pipecolic acid that was left as unmetabolized during the cultivation. The initial amount of D-pipecolic acid was 15 g/l.

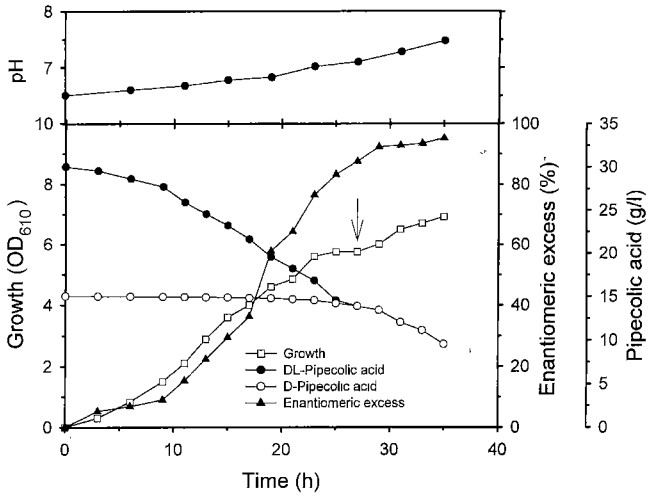

Fig. 1. Time course for the resolution of DL-pipecolic acid by *Pseudomonas* sp. PA09. Cultivations were carried out at 33°C in the medium (pH 6.5) containing 3% DL-pipecolic acid, 0.12% NH_4NO_3, 0.1% yeast extract, and other elements as indicated in Material and Methods. The arrow indicates the time when the second growth began.

in the absence of L-pipecolic acid. In a recent study, Mochizuki *et al.* [15] reported that *Alcaligenes* sp. assimilated L-pipecolic acid from 5% DL-pipecolic acid after 72 h cultivation, producing D-pipecolic acid with the e.e. and the residual D-pipecolic acid at 99% and 95%, respectively. Their results showed the highest yield among previous studies (reference 15 and therein) until our present study: The productivity with *Pseudomonas* PA09 in the present study was higher than that with *Alcaligenes* sp., because the cultivation time with *Pseudomonas* sp. was one-third of that with *Alcaligenes* sp. at a slightly lower concentration.

Overall, the results suggest that an optically pure chemical, such as D-pipecolic acid, can be prepared from the racemate by applying a simple fermentation process, if an appropriate strain, which preferentially metabolizes the undesired enantiomer, is obtained. Such process is very economical since DL-pipecolic acid can be chemically synthesized from picolic acid which is an inexpensive chemical [15].

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