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Review

Industrial biotransformations for the production of D-amino acids

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Abstract

Optically pure D-amino acids are industrially manufactured by biotransformations of cheap starting materials produced by chemical synthesis or fermentation in combination with the development of enzyme catalysts suitable for the starting materials. DL-Alaninamide, an intermediate of the chemical synthesis of DL-alanine, was efficiently converted to D-alanine by stereoselective hydrolysis with a D-isomer specific amidohydrolase produced by *Arthrobacter* sp. NJ-26. The total utilization system of DL-alaninamide for the production of optically pure D- and L-alanine was constructed by stereospecific amidohydrolases. On the other hand, D-amino acids were also produced from corresponding L-isomers, which are efficiently manufactured by fermentation. D-Glutamic acid was produced from L-glutamic acid. L-Glutamate was converted to the DL-form by the recombinant glutamate racemase of *Lactobacillus brevis* ATCC8287. Then L-glutamate in a racemic mixture was selectively decarboxylated to γ-aminobutyrate by the L-glutamate decarboxylase of *E. coli* ATCC11246. As a result of successive enzymatic reactions, D-glutamate was efficiently produced from L-glutamate by a one-pot reaction. D-Proline was produced by the same strategy from L-proline using the recombinant proline racemase of *Clostridium sticklandii* ATCC12262. In this case, L-proline was degraded by *Candida* sp. PRD-234. The strategy from L-amino acids to D-amino acids could be applicable to the manufacture of many D-amino acids. © 1998 Elsevier Science B.V.

Keywords: D-Amino acids; Asymmetric hydrolysis; Chiral resolution; Stereospecific amidohydrolase; Amino acid racemase; L-Glutamate decarboxylase

1. Introduction

D-Amino acids are becoming important as intermediates for the production of pharmaceuticals, food additives and agrochemicals. D-*p*-Hydroxyphenylglycine and D-phenylglycine are utilized for the synthesis of penicillin derivatives [1–3]. D-Cysteine and D-aspartic acid are important as side-chains of beta-lactam antibiotics and D-valine is for insecticides [4,5]. D-Alanine is used as a starting material for a synthetic sweetener [6,7]. D-Glutamate and D-proline are used for synthetic pharmaceuticals. Although the production of D-amino acids is currently of great interest, there has been no known industrial manufacture of D-amino acids except for D-*p*-hydroxyphenylglycine and D-phenylglycine.

To establish the industrial manufacture of D-amino acids, the following points should be considered: (1) the availability of starting materials, (2) the optical purity of products and (3)

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productivity. Under these criteria, three possible methods, i.e., chemical synthesis, fermentation and an enzymatic method, are compared for the industrial manufacture of D-amino acids.

DL-Amino acids such as DL-methionine and DL-alanine are industrially manufactured by chemical synthesis from the corresponding aldehyde and cyanic acid. D-Amino acids can be produced by chiral resolution of these DL-amino acids. On the other hand, asymmetric synthesis of D-amino acids from chiral or prochiral starting materials have been known. However, chemical methods are not suitable for a large scale production of D-amino acids at the moment because of low yield and high cost.

Microorganisms contain several D-amino acids, such as D-alanine, D-glutamic acid, D-aspartic acid, D-proline, D-leucine, D-isoleucine, D-valine, D-tryptophane, D-phenylalanine and D-ornithine as components of the cell wall peptide glycan or peptide antibiotics [8–10]. These D-amino acids are considered to be produced from corresponding L-amino acids or α-keto-acids in biological systems by amino acid racemases or D-stereospecifc transaminases in combination with amino acid racemases. Most of the L-amino acids are efficiently manufactured by fermentation, but the D-amino acids are hardly produced by fermentation, with a few exceptions, because it is difficult to obtain high optical purity and productivity. Takeuchi et al. reported D-alanine fermentation by a D-cycloserine resistant mutant of *Brevibacterium lactofermentum* [11]. However, the optical purity of the D-alanine produced was relatively low because of an alanine racemase activity.

Enzymatic methods are most plausible for the industrial manufacture of D-amino acids in regard to the optical purity and productivity of D-amino acids as D-*p*-hydroxyphenylglycine and D-phenylglycine are produced from DL-hydantoins. The enzymatic production of D-amino acids is classified into three categories based on the starting materials (Table 1). (1) DL-Amino acids: it has been reported that D-amino acids were obtained by stereoselective degradation of L-amino acids of DL-amino acids [5,12]. Asymmetric hydrolysis of *N*-acyl-DL-amino acids with D-amino acylase is also reported for the production of D-amino acids [13–19]. (2) Synthetic intermediates of DL-amino acids: D-amino acids can be produced by D-specific hydrolysis of 5-substituted DL-hydantoins [1,20–35]. Asymmetric hydrolysis of DL-amino acid amides is also reported for the production of D-amino acids by D-amino acid amidohydrolases [36–39]. (3) Prochiral compounds: D-amino acid transaminase is used to produce D-amino acids from corresponding α-keto acids. Soda et al. reported D-amino acid production from α-keto acids by the system constructed with D-amino acid transaminase, alanine racemase, alanine dehydrogenase and formate dehydrogenase [40–47].

From an industrial standpoint of view, availability of cheap starting materials and the development of enzyme catalysts suitable for the starting materials are most important. This review describes our efforts to develop the en-

Table 1
Enzymatic methods for the production of D-amino acids

Category	Starting materials	Enzymes or methods	Refs.
DL-amino acids	DL-amino acids	degradation of L-amino acids	[5,12]
	N-acyl-DL-amino acids	D-amino acylase	[14–19]
Synthetic intermediates	DL-hydantoin	D-hydantoin hydrolase and N-carbamoyl-D-amino acid hydrolase	[1,20–35]
	DL-amino acid amides	D-amidase	[36–39]
Prochiral substrates	α-keto acids and L-amino acids	D-transaminase and amino acid racemase	[40–47]

zyme catalysts for establishing the industrial manufacture of D-amino acids.

2. Stereoselective hydrolysis of synthetic intermediate

2.1. Screening of stereoselective amidohydrolases

DL-Alanine is industrially synthesized from acetaldehyde and cyanic acid by the Strecker reaction to form DL-2-aminopropionitrile, which is then hydrolyzed to DL-alanine. DL-Alaninamide was chosen as a starting substrate for an industrial manufacture of D-alanine because of the following reasons: (1) DL-alaninamide can be easily isolated on a large scale as an intermediate of the chemical synthesis of DL-alanine and is therefore very cheap. (2) A kinetic resolution system can be constructed for the production of optically pure D- and L-alanine from DL-alaninamide (Fig. 1). Although stereoselective amidohydrolases are required to construct the kinetic resolution system of DL-alaninamide, there have been few reports of the stereoselective amidohydrolases on DL-alaninamide. Asano et al. reported the D-isomer selective hydrolysis of D-alaninamide by the D-aminopeptidase of *Ochrobactrum anthropi* [36–39,48]. Dotani et al. also reported D-stereoselective hydrolysis of D-alaninamide by D-isomer selective amidohydrolases of *Achromobacter cycloclas*, *Alcaligenes faecalis* and *Kurthia zophii* [49]. L-Alaninamide selective amidohydrolases from

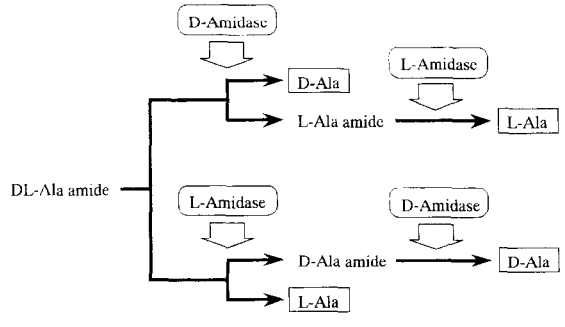

Fig. 1. Utilization of DL-alaninamide for the production of D- and L-alanine.

Table 2
Properties of D-alaninamide specific amidohydrolase

Molecular mass		51 000 Da
Subunit		monomer
Cofactor		none
Inducers		D-, L- and DL-alaninamide
Substrate specificity	(substrate)	(relative activity)
	D-alaninamide	100
	L-alaninamide	0.67
K_m (mM)	D-alaninamide	4.19
	L-alaninamide	26.1
V_{max} (μmol/mg per min)		1380
Optimal pH		7.5
Optimal temperature		45°C

Brevibacterium sp., *Ochrobactrum anthropi*, *Pseudomonas putida*, *Arthrobacter* sp. and *Corynebacterium* sp. were also reported [36–38,49,50]. However, these enzymes were not sufficient as tools for the industrial manufacture of D- and L-alanine because of low activities and stereoselectivities. Thus, we started the screening of stereoselective amidohydrolases that are suitable for the industrial process.

D-Alaninamide selective amidohydrolases can be found in microorganisms which grew on the medium containing 2 g/l of DL–aninamide as a sole source of nitrogen and 0.5 g/l of D-cycloserine since D-cycloserine, which is an inhibitor of alanine racemase, makes microorganisms require D-alanine for their growth. The isolated strains were aerobically cultured in the medium containing 2 g/l of DL–aninamide and their ability to generate D-alanine from DL-alaninamide was evaluated by whole cell reaction. As a result of screening, *Arthrobacter* sp. NJ-26 was isolated from active sludge [51]. The cellular activity of D-alaninamide selective amidase (D-amidase) of *Arthrobacter* sp. NJ-26 was about 800 μmol/min per g wet cells. The D-amidase activity was induced by only D-, L- and DL-alaninamide. The cellular L-alaninamide amide hydrolase (L-amidase) activity and alanine racemase activity were also detected at 1.3% and less than 0.5% of D-amidase activity, respectively. The D-amidase produced by *Arthrobacter* sp. NJ-26 was purified to near homogeneity and characterized (Table 2) [51].

The D-amidase is highly specific for D-alaninamide. The K_m for D-alaninamide was 4.19 mM and V_{max} for D-alaninamide was 1,380 μmol/mg per min. The purified enzyme has also a weak L-amidase activity, however, V_{max} for L-alaninamide was 0.67% of that for D-alaninamide and the K_m for L-alaninamide was 26.1 mM.

L-Amidases can also be found in microorganisms which grew on the medium containing L-alaninamide as a sole source of nitrogen. The isolated strains were aerobically cultured in the medium containing 2 g/l of DL-alaninamide and their ability to generate L-alanine from DL-aninamide was evaluated by the whole cell reaction. As a result of screening, *Rahnella* sp. L-5 was isolated from soil. The cellular activity of strain L-5 was 468 μmol/min per g wet cells. The enzyme activity was induced by L-alaninamide but was not induced by D-alaninamide. Cellular D-amidase and the alanine racemase of strain L-5 were 4.0 and 0.7% of L-amidase activity, respectively.

2.2. *Kinetic resolution of DL-alaninamide*

D-Alanine production from DL-alaninamide was examined by the whole cell reaction with *Arthrobacter* sp. NJ-26 to optimize the conditions for D-alanine production. The optical purity of D-alanine produced from DL-alaninamide was affected by the activities of alanine racemase and L-amidase. The optimal pH values for D-amidase and alanine racemase activities were 7.5 and 8.0, respectively. Alanine racemase was not active at a pH below 7.0, however, D-amidase still retained its activity at pH 6.5 (Fig. 2A). Therefore, a high optical purity of D-alanine was obtained by the reaction between pH 6.5 and 7.0. On the other hand, L-amidase activity of the D-amidase was affected by the concentration of DL-alaninamide. When L-alaninamide was used as a substrate, L-amidase activity was not affected at all. On the contrary, L-amidase activity was severely inhibited by DL-alaninamide at the concentration of more than 1.7 M (150 g/l) of L-aninamide (Fig. 2B). No substrate inhibition of the D-amidase activity was observed up to 3.4 M (300 g/l) of DL-alaninamide. As a result of optimization, the condition for D-alanine production was determined as follows; 38°C, pH 6.8 and the substrate DL-alaninamide at a concentration of more than 2.4 M (210 g/l). The whole cell reaction with 2.4 M of DL-alaninamide and 5 g/l of wet cells of NJ-26 finished up in 10 h to give 1.2 M (105 g/l) of D-alanine. The optical purity of D-alanine produced was higher than 99% ee and L-alaninamide remained in the reaction mixture quantitatively.

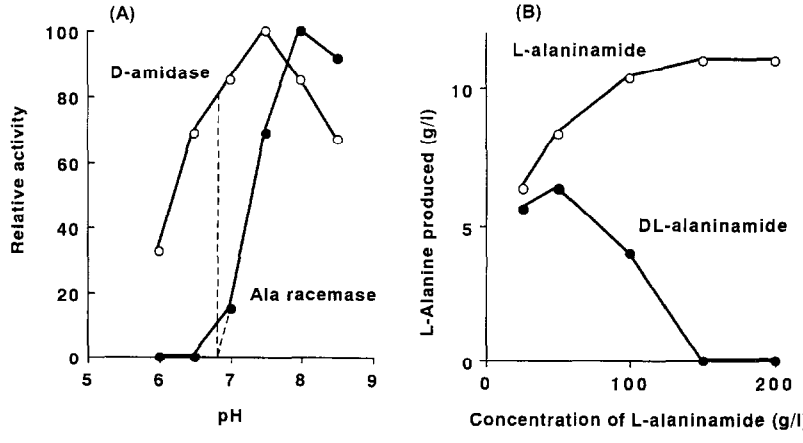

Fig. 2. Effect of pH and substrate concentration on D-amidase, L-amidase and alanine racemase activities. (A) Effect of pH on D-amidase and alanine racemase activities. (B) Effect of substrate on L-amidase activity.

D-Alanine can also be produced from D-alaninamide which remains in the reaction mixture after stereoselective hydrolysis of L-alaninamide of DL-alaninamide. Then, the stereoselective hydrolysis of L-alaninamide from DL-alaninamide was examined by whole cell reaction with *Rahnella* sp. L-5. The optimum pH and temperature of the reaction were determined as being 7.0 and 35°C, respectively. The D-amidase activity of strain L-5 was inhibited by L-alaninamide and no substrate inhibition on the L-amidase activity was observed up to 2 M of L-alaninamide. The whole cell reaction with 20 g/l of wet cells of strain L-5 and 3.4 M of DL-alaninamide finished up quantitatively in 24 h to give 1.7 M of L-alanine. The optical purity of L-alanine was more than 98% ee and D-alaninamide remained in the reaction mixture quantitatively.

2.3. Construction of the total system for DL-alaninamide utilization

Although the enzymatic production of D-alanine from DL-alaninamide was successfully achieved on a large scale by *Arthrobacter* sp. NJ-26, it was difficult to obtain the cells with maximum D-amidase activity because of the rapid hydrolysis DL-alaninamide added to the medium as an inducer. Thus, a D-amidase constitutive mutant was isolated from NJ-26 to overcome this drawback of the culture. 2-Cyanoacetamide is a weak substrate of the D-amidase but has no inducing activity on the enzyme. A mutant strain CY-7-J2, that grew on the agar plate containing 2-cyanoacetamide as a sole source of nitrogen, was isolated as a D-amidase constitutive mutant [52]. The enzyme properties of CY-7-J2 were the same as those of NJ-26. The cellular D-amidase activity of CY-7-J2 was 1.8 times higher than that of NJ-26. The whole cell reaction with 5 g/l of wet cells of CY-7-J2 and 2.4 M of DL-alaninamide finished in 5 h to give 1.2 M of D-alanine.

A large scale production of L-alanine from DL-alaninamide was also effectively achieved by *Rahnella* sp. L-5. In this case, the pH of the whole cell reaction should be kept lower than 7.0 to obtain high optical purity. A L-amidase constitutive mutant can be obtained in the same manner as described above.

It is possible to construct an efficient system for DL-alaninamide utilization by *Arthobacter* sp. CY-7-J2 and *Rahnella* sp. L-5 (Fig. 1). Starting from DL-alaninamide and CY-7-J2, optically pure D-alanine and L-alaninamide were obtained efficiently. L-Alaninamide was then converted to L-alanine by the strain L-5. Starting from the strain L-5, L-alanine and D-alaninamide were also obtained. D-Alaninamide was then converted to D-alanine by CY-7-J2. Furthermore, L- and D-alaninamide are themselves useful as chiral starting materials.

3. Production of D-amino acids from L-amino acids

3.1. Strategy for the production of D-amino acids from L-amino acids

Since most L-amino acids are manufactured inexpensively by fermentation, L-amino acids should be considered as starting materials for

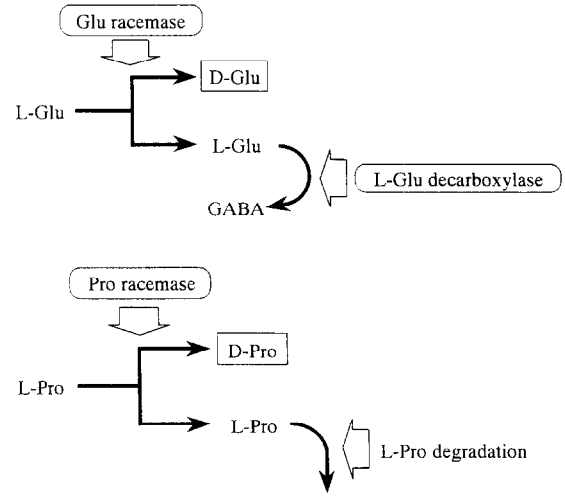

Fig. 3. Strategy for the production of D-amino acids from L-amino acids.

the enzymatic production of D-amino acids. The first step in the production of D-amino acids from L-amino acids is the racemization of L-isomers to the DL-form. L-Isomers in racemic mixtures are then selectively degraded enzymatically and D-amino acids remain in reaction mixtures. Although the theoretical yield of D-amino acids from L-amino acids is 50% according to this strategy, it is still possible to apply this strategy to the industrial manufacture of D-amino acids, considering the availability and cheapness of L-amino acids produced by fermentation. In Section 3.2 enzymatic production of D-glutamic acid and D-proline from corresponding L-isomers is described (Fig. 3).

3.2. Screening of amino acid racemases and the gene cloning

Glutamate racemases have been reported to occur exclusively in lactic acid bacteria, i.e., *Lactobacillus fermenti* [53], *Pediococcus pentosaceus* [54,55], *L. arabinosus* [56] and *L. plantarums* [57]. We therefore have screened glutamate racemases, which are suitable for the industrial manufacture of D-glutamate, among lactic bacteria. Glutamate racemase activities were detected in 64 strains among 70 lactic acid bacteria examined, however, the reactivities of most of these glutamate racemases tended to decline or saturate at concentrations higher than 150 mM of L-glutamate. Among them, the reactivity of the enzyme of *L. brevis* ATCC8287 increased almost proportionally according to the increase of the concentration of L-glutamate. This strain racemized 66% of L-glutamate to form 165 mM of D-glutamate by whole cell reaction for 4 h even at the concentration of 500 mM L-glutamate [58]. However, *L. brevis* ATCC8287 is not suitable for the industrial process because it is very difficult to obtain a sufficiently large cell mass. To improve this drawback, the glutamate racemase gene of *L. brevis* ATCC8287 was cloned in *E. coli* [59].

The glutamate racemase gene of *L. brevis* ATCC8287 was cloned in *E. coli* TM93 by complementation of the L-glutamate requirement of TM93 on a minimum agar plate containing D-glutamate. Two types of plasmids, pGAR1 and pGAR2, were isolated from the transformants. Both plasmids contained an identical 2.8 kb of inserted DNA at the *Hind* III site of pBR322. The glutamate racemase activities of transformants were approximately 150 times higher than that of *L. brevis* ATCC8287 (Fig. 4). The glutamate racemase gene encodes a protein of 276 amino acids (828 b). The glutamate racemase was purified to homogeneity from *E. coli* TM93/pGAR1 and characterized (Table 3). The enzyme is a monomer enzyme and its molecular weight was 29,426. The enzyme seemed to be co-factor independent. The V_{max} was 216 μmol/min per mg protein at pH 8.5 and 37°C. The enzyme did not catalyze the racemization of any amino acids other than glutamate and the K_m values for L- and D-glutamate were almost equal. No substrate inhibition was observed up to 2.0 M (300 g/l) of L-glutamate.

Proline racemase was reported only in

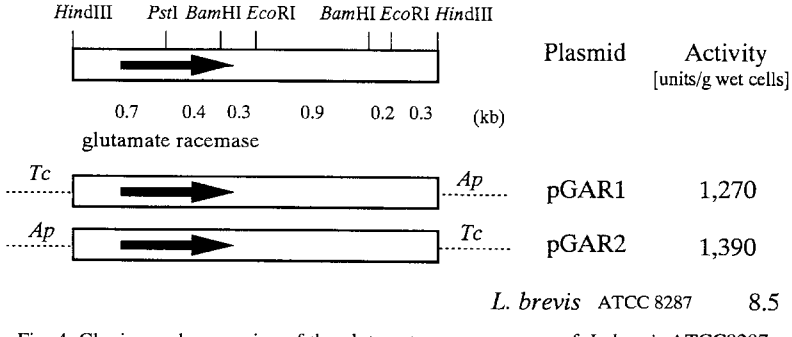

Fig. 4. Cloning and expression of the glutamate racemase gene of *L. brevis* ATCC8287.

Table 3
Properties of glutamate racemase and proline racemase

	Glutamate racemase	Proline racemase
Molecular mass	29426 Da	36825 Da
Subunit	monomer	dimer
Cofactor	none	none
Substrate specificity	D- and L-Glu	D- and L-Pro
K_m (mM)	D-Glu 5.94	D-Pro 10.6
	L-Glu 6.39	L-Pro 17.4
V_{max} (μmol/mg per min)	D-Glu 208	D-Pro 1,248
	L-Glu 216	L-Pro 1,448
Optimal pH	8.5	8.0
Optimal temperature	37°C	55°C

Clostridium sticklandii ATCC12662 [60–68,76]. We screened proline racemases among 20 *Clostridium* strains and found activities in *C. scatologenes* KY20022 and *Clostridium* sp. KY20005 as well as *C. sticklandii* ATCC12662. After examination of the industrial applicability of these proline racemase activities, *C. sticklandii* ATCC12262 was chosen since the activity was higher and more stable than those of the other two strains and high concentration of L-proline up to 3.48 M (400 g/l) did not inhibit the activity. *C. sticklandii* ATCC12662 is not suitable for the industrial process because of the same reason as in the case of *L. brevis* ATCC8287. The proline racemase gene of *C. sticklandii* ATCC12662 was also cloned in *E. coli* [69].

The proline racemase gene of *C. sticklandii* ATCC12262 was cloned in *E. coli* HB101 by the complementation of the L-proline requirement of HB101 on a minimum agar plate containing D-proline. A plasmid pPR1, which contained 9.8 kb of inserted DNA at the *Hin*dIII site of pBR322, was recovered. As a result of subcloning, a transformant containing pPR3 showed 23 times higher proline racemase activity than that of *C. sticklandii* ATCC12662 (Fig. 5). The recombinant plasmid pPR3 contained a 1.0 kb *Hin*dIII–*Eco*RI fragment of the 9.8 kb *Hin*dIII fragment of pPR1. A promoter in the 1.0 kb *Hin*dIII–*Eco*RI fragment caused high expression of proline racemase in *E. coli*. The proline racemase gene encodes a protein of 335 amino acids (1005 b). The proline racemase was purified to homogeneity from *E. coli* HB101/pPR3 and characterized (Table 3). The enzyme consisted of two identical subunits and the molecular weight of a subunit was 36,825. The enzyme required no co-factor for the activity. The V_{max} was 1,448 μmol/min per mg protein at pH 8.0 and 55°C. K_m values for L- and D-proline were almost equal. The enzyme did not catalyze racemization of amino acids other than proline. No substrate inhibition was observed up to 1.74 M (200 g/l) of L-proline.

3.3. Selective degradation of L-amino acids in racemic mixtures

Degradation of L-glutamate is necessary for the enzymatic resolution of DL-glutamate at the second step of D-glutamate production. Among

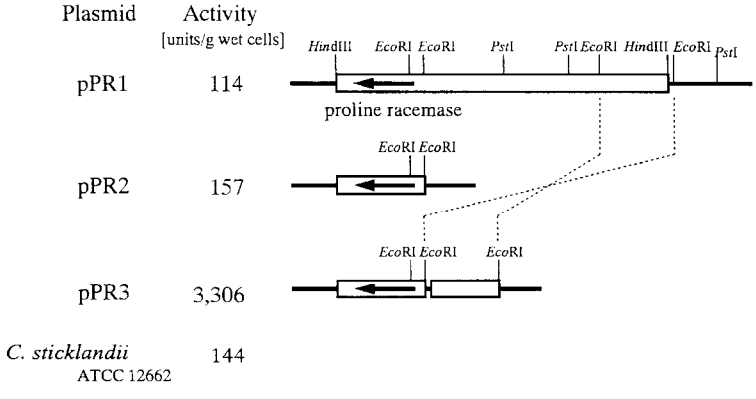

Fig. 5. Cloning and expression of the proline racemase gene of *C. sticklandii* ATCC12662.

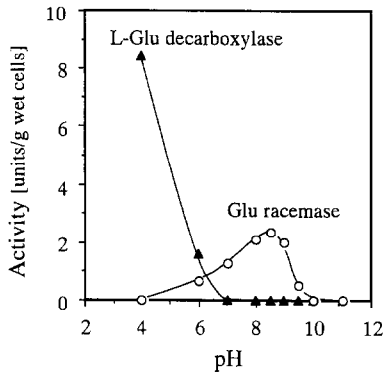

Fig. 6. Effect of pH on glutamate racemase and glutamate decarboxylase activities.

several enzymes that act on L-glutamate, i.e., glutamate decarboxylase, dehydratase, dehydrogenase and oxidase, L-glutamate decarboxylase was chosen as the enzyme for the enzymatic resolution of DL-glutamate because the reaction is irreversible with high stereoselectivity. Although *L. brevis* ATCC8287 has L-glutamate decarboxylase and it is possible to produce D-glutamate from the L-isomer by a one-pot reaction with this strain [58], the strain is not suitable for the industrial process since it is difficult to obtain a large cell mass. Thus, we screened L-glutamate decarboxylase activities of *E. coli* strains since *E. coli* strains has been reported to produce relatively high activities of L-glutamate decarboxylase [70–74]. As a result of the screening, *E. coli* ATCC11246 was selected as a producer of L-glutamate decarboxylase [75]. The cellular activity of the L-glutamate decarboxylase of *E. coli* ATCC11246 was 91 μmol/min per g wet cells, which was more than 11 times higher than that of *L. brevis* ATCC 8287.

Microorganisms for L-proline degradation were also screened. Microorganisms which grew on the medium containing 2 g/l of L-proline as a sole source of carbon and nitrogen were selected from soil and examined for L-proline degradation. As a result of screening, *Candida* sp. PRD-234 was selected. The optimal pH of L-proline degradation in the strain PRD-234 was 5.5 and 7.5. The results suggested that the strain PRD-234 has two pathways for the degradation of L-proline. The optimal temperature for the reaction was 35°C, but a temperature of 30°C was more suitable for a long time reaction. The strain PRD-234 also had D-proline degradation activity when D-proline was used as a substrate for the reaction. However, the activity of D-proline degradation was not detected when L-proline existed. Therefore, if the reaction was stopped simultaneously with the disappearance of L-proline, the strain PRD-234 was able to degrade L-proline of DL-proline completely without any loss of D-proline.

3.4. Production of D-glutamic acid and D-proline

The production of D-glutamate from L-glutamate was examined by *E. coli* TM93/pGAR1 and *E. coli* ATCC11246 cells which were permeabilized with 2% (v/v) of toluene. The optimum pH for the racemization of L-glutamate by the TM93/pGAR1 strain was pH 8.5 (Fig. 6). Under this condition, 1.36 M (200 g/l) of L-glutamate was completely racemized within 24 h at 37°C with 2.5 g/l of wet cells of TM93/pGAR1. The optimum pH for the degradation of L-glutamate by *E. coli* ATCC11246 was below 4.0 (Fig. 6). L-Glutamate of 0.68 M DL-glutamate was completely decarboxylated to γ-aminobutyrate (GABA) at pH 4.2 with 5 g/l of wet cells of strain ATCC11246 within 10 h at 37°C. Therefore, an efficient production of D-glutamate from L-glutamate was achieved by two successive whole cell reactions with a pH decrease. Starting at 0.68 M of L-glutamate, L-glutamate was completely racemized by the reaction with 3 g/l wet cells of *E. coli* TM93/pGAR1 at 37°C and pH 8.5 for 5 h and 0.34 M of D-glutamate was produced (Fig. 7). After the pH of the reaction mixture was decreased to 4.2, ATCC11246 cells were added to the reaction mixture of 5 g/l of wet cells. After 10 h, L-glutamate was completely converted to GABA and 0.34 M (50

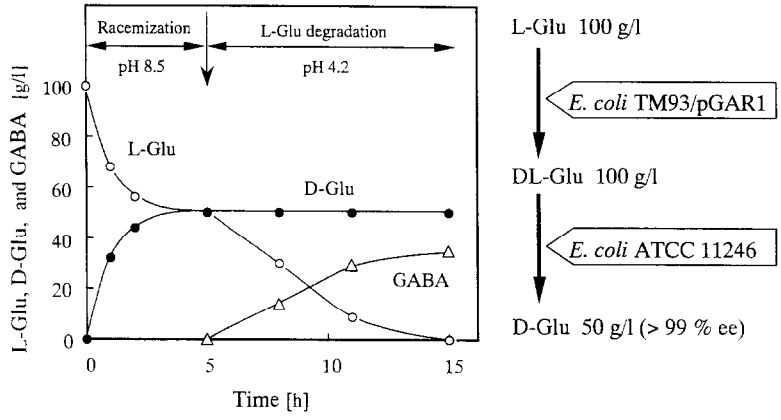

Fig. 7. Production of D-glutamate from L-glutamate.

g/l) of D-glutamate remained in the reaction mixture. The optical purity of the D-glutamate isolated was more than 99% ee.

Conditions for the production of D-proline from L-proline were examined by *E. coli* HB101/pPR3 and *Candida* sp. PRD-234. The optimal pH and temperature for the racemization of L-proline by *E. coli* HB101/pPR3 were pH 6.2 and 37°C, respectively. The L-proline of 0.87 M (100 g/l) was completely racemized within 24 h under these condition with 1 g/l wet cells of a strain HB101/pPR3 which was permeabilized with 1% (v/v) toluene (Fig. 8A). After the racemization, the reaction mixture was heated to 120°C for 20 min to inactivate the proline racemase. The optimal pH and temperature for L-proline degradation with strain PRD-234 were pH 6.0 and 30°C, respectively. Since the cells degrade L-proline under aerobic conditions, the reaction required agitation at 600 rpm and aeration of 1 l/l/min. The dissolved oxygen level of the reaction mixture sharply rose when L-proline was completely consumed. PRD-234 cells of 40 g/l metabolized completely 0.43 M (50 g/l) of L-proline within 40 h at pH 6.0 and 30°C (Fig. 8B). Since the cell mass of *Candida* sp. PRD-234 increased during the L-proline degradation, the degraded L-proline might be metabolized to cell mass and carbon dioxide. The optical purity of D-proline remaining in the reaction mixture was 99.5% ee. The yield of D-proline from L-proline was 47%.

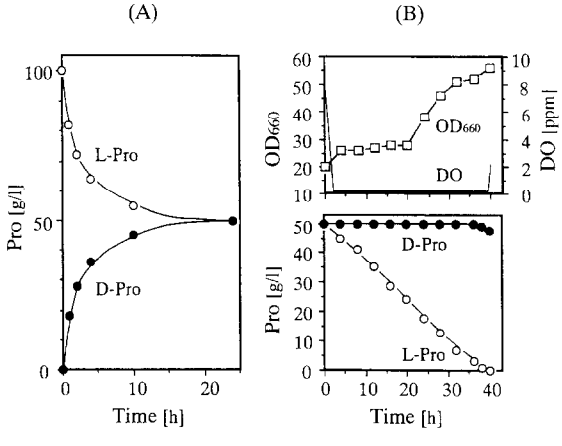

Fig. 8. Production of D-proline from L-proline. (A) Racemization of L-proline with *E. coli* HB101/pPR3. (B) Degradation of L-proline with *Candida* sp. PRD-234. DO: Concentration of dissolved oxygen in the reaction mixture.

4. Discussion

Enzymatic methods are most plausible for the industrial manufacture of D-amino acids as described above, but several factors should be considered in establishing the industrial process of D-amino acids. Among them, the availability of starting materials is most important. A number of enzymatic methods were reported as shown in Table 1, however, the number of substrates that are available on an industrial

scale is limited. Under these criteria, synthetic intermediates of DL-amino acids and L-amino acids produced by fermentation would be most important starting materials for the production of D-amino acids. D-Methionine can be produced from the synthetic intermediate using enzymatic methods as well as D-*p*-hydroxyphenylglycine and D-phenylglycine, since the DL-form is produced from DL-hydantoin using the synthetic method [1–3]. This is also the case for the production of D-phenylalanine. However, the applicability of the synthesis from synthetic intermediates to D-amino acids is restricted as the number of amino acids produced using the synthetic method is limited. On the other hand, the synthesis from L-amino acids has a broader applicability to the production of D-amino acids than the synthesis from synthetic intermediates since most of the L-amino acids are manufactured inexpensively by fermentation. Despite the drawback of the theoretical yield of D-amino acids from L-amino acids being 50%, this method is still practical for the industrial manufacture of D-amino acids considering the cheapness of L-amino acids produced by fermentation. Furthermore, there are many known amino acid racemases [77,78]. In fact, several D-amino acids such as D-lysine and D-histidine were produced using this method, as well as D-glutamate and D-proline, in our laboratory (unpublished results).

The theoretical yield of D-amino acids from the racemates is 50% whether the starting materials are synthetic compounds or fermentation products. Therefore, some tricks to racemize the remaining half are required to obtain the 100% yield. Huh et al. reported the synthesis of L-proline from racemate by coupling enzymatic enantiospecific oxidation and chemical non-enantiospecific reduction [79]. According to their report, D-proline of DL-proline is converted enzymatically to pyrroline-2-carboxylic acid which is then reduced chemically to DL-proline. It is not impossible to obtain a 100% yield of the products from racemates.

The development of enzyme catalysts suitable for the starting materials is also very important. A combination of good screening techniques and recombinant DNA technology makes it possible to obtain the enzyme catalysts that have strict stereoselectivities and high activities. Further studies are expected to produce various D-amino acids by enzymatic methods.

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