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Isolation of a High-Yield Mutant Strain for L-Proline Production and Its Fermentation Conditions

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Abstract L-Proline-producing mutant strains were developed by exposing L-glutamic acid-producing bacteria to *N*-metyl-*N*-nitro-nitrosoguanidine and UV irradiation. A L-histidine auxotroph of *Corynebacterium acetoacidophilum* RYU3161 (KCTC 0616BP), which was resistant to sulfaguanidine and proline analogs (DHP, AZC, TAC), was isolated. The activity of the mutant strain's γ-glutamyl kinase was 45% higher than that of the parent strain. The optimum level of L-histidine for production of L-proline was 0.16 g/l. In a 5-l jar fermenter, the mutant strain produced L-proline at a high concentration (35 g/l) level within 48 h of cultivation.

Key words: L-proline, L-histidine auxotroph, sulfaguanidine resistant mutant, proline analog resistant mutant, γ-glutamyl kinase

In order to improve amino acids production yields, many novel approaches including molecular biological means are being widely used in industry today [9, 31]. However, conventional mutation is still a very powerful technique to develop industrial strains [11].

L-proline (2-pyrrolidinecarboxyic acid) is a pharmaceutically important non-essential amino acid and a useful additive for drugs and feed [15]. It is one of the cyclic aliphatic amino acids, which helps maintain and strengthen the heart. For this reason, it has a sizable world market [1]. L-proline is synthesized from L-glutamic acid via three enzymatic reactions and one spontaneous cyclization in microorganisms [3, 4, 22-25]. The biosynthetic pathway of L-proline from mutant strains of *Escherichia coli* was first reported by Vogel and Davis, and the formation of L-proline from L-glutamic acid has been demonstrated [22, 24].

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The formation of γ-glutamyl kinase, a key enzyme in the first step of the L-proline biosynthetic pathway, is repressed by L-proline in L-glutamic acid-producing bacteria [3, 4, 7, 23, 29]. Amino acid high-producing strains have been selected from amino-acid-analog resistant mutants [5]. Therefore, derivation of mutant strains resistant to proline analogs is a prerequisite for the overproduction of L-proline [13, 26, 28].

It was reported that 12 g/l of L-proline was produced from 10% glucose by an L-isoleucine auxotroph of *Brevibacterium flavum* [27, 30]. L-Proline was also accumulated by various mutants of other L-glutamic acid-producing bacteria; the L-histidine auxotroph of *Brevibacterium sp.*, a phenylalanine and tyrosine auxotroph of *Corynebacterium mealssecola,* and an isoleucine auxotroph of *C. glutamicum* [2, 10, 14, 18]. However, the yields of L-proline from these strains were relatively low and not appropriate for industrial use.

Biosynthesis of L-proline was reported to be improved by adding an excess supply of ATP to a key enzyme, γ-glutamyl kinase [21]. This observation indicated that the fermentation yield of L-proline in industrial processes was significantly improved by increasing the intracellular ATP level. Increased ATP levels made the microorganism more resistant to analogs of the compounds related to purine biosynthesis. Therefore, mutants showing an enhanced biosynthetic activity of L-proline could be found among those resistant to sulfonamides [21].

In this study, in order to obtain high yield strains for L-proline, mutants have been screened from L-glutamic acid-producing bacteria by deriving L-isoleucine or L-histidine auxotrophs and mutants resistant to sulfaguanidine. From these strains, high-yield mutants were further derived by selecting strains resistant to proline analogs.

The optimal fermentation conditions for the L-proline production using this new strain was investigated in shake-

flasks and jar fermenters. The activity of γ-glutamyl kinase of the mutant strain was also measured to identify the correlation between the enzyme activity and L-proline production.

MATERIALS AND METHODS

Microorganisms

The microorganisms used in this study were as follows; *Brevibacterium lactofermentum* ATCC 13869, *Corynebacterium acetoacidophilum* ATCC 13870, *Brevibacterium flavum* ATCC 15940, and *Corynebacterium glutamicum* ATCC 15942. *B. lactofermentum* ATCC 13869 and *C. acetoacidophilum* ATCC 13870 were wild-type strains. *B. flavum* ATCC 15940 and *C. glutamicum* ATCC 15942 were L-isoleucine and L-histidine auxotrophs, respectively. These parent strains produced only very small amounts of L-proline, as shown in Table 1. High-yield mutant strains were therefore derived from these parent strains by applying the conventional mutation techniques, such as treating with NTG and/or UV irradiation.

Culture Media and Conditions

YPD medium was used for the seed culture and M9 medium as a minimal medium, with some modifications [17]. M9 medium was supplemented with 100 μg/ml of L-histidine or L-isoleucine, whenever needed. The medium composition used in the shake-flask culture was (in g/l of distilled water); glucose, 100; $(NH_4)_2SO_4$, 30; KH_2PO_4, 3; $MgSO_47H_2O$, 1; yeast extract, 7; $CaCO_3$, 20 (sterilized separately); and 0.1% (v/v) of the stock solution containing 100 μM of various trace minerals. The pH of the initial medium was adjusted to 7.3 with 4 N NaOH solution. The main fermentation was carried out in a 5-l jar fermenter (Model KFC MK-250, Korea Fermentor Co., Ltd., InChon, Korea) with a 2-l working volume containing 10% glucose, 3% $(NH_4)_2SO_4$, 0.7% yeast extract, 0.3% KH_2PO_4, and 0.1% (v/v) of the mineral stock solution. Temperature and pH were controlled at 30°C and 7.0 with 4 N NH_4OH, respectively. Air was supplied at an aeration rate of 0.5-1 vvm. The agitation speed was controlled at 600-800 rpm based on the DO level measurement.

Table 1. Characteristics of the parent strains in this study.

Strains	Characteristics*	L-proline produced (g/l)
Brevibacterium lactofermentum ATCC13869	wild-type	0
Corynebacterium acetoacidophilum ATCC13870	wild-type	0
Brevibacterium flavum ATCC15940	Ile^-	1.5
Corynebacterium glutamicum ATCC15942	His^-	2

*Ile^-: L-isoleucine auxotroph.
His^-: L-histidine auxotroph.

Analytical Methods

Cell growth was monitored by the optical density (OD) at 600 nm using a spectrophotometer (Model UVICON 930, Kontron Instruments, Italy). The OD value was converted to dry cell weight (DCW) by using a calibration curve. The glucose concentration was measured using a Glucose & Lactate Anaylzer (Model 2300 STAT, Yellow Springs Instruments, U.S.A.). L-Proline concentration was determined by using the ninhydrin reaction [7]. γ-Glutamyl kinase was assayed by a colormetric method based on that of Baich [3, 28, 29].

RESULTS AND DISCUSSION

Sensitivity of L-Glutamic Acid-Producing Bacteria to Sulfaguanidine (SG) and Proline Analogs (DHP, AZC, TAC)

For isolating the mutant strains, the sensitivities of L-glutamic acid-producing bacteria to the sulfaguanidine (SG) and proline anaolgs were first investigated. SG is widely used as an antimicrobial agent, because it inhibits the growth of many bacteria by preventing the formation of folic acid in microorganisms. It also prevents purine biosynthesis by indirectly inhibiting the formation of 5-phosphoribosyl-4-carboxamide-5-aminoimidazole. Therefore, the mutant strains resistant to SG are regarded as possessing enhanced activity of ATP biosynthesis to overcome such inhibitory effects. Since its growth was almost completely inhibited by 400 μg/ml of SG, mutant strains were isolated as the colonies appeared on minimal agar plates containing the same concentration level of SG. Figure 1 shows the sensitivities of one of the mutant strains, RYU3161, and the parent strain, *C. acetocidophilum* ATCC 13870, against SG.

The mutant strain RYU3161 was highly resistant to 400 μg/ml of SG, but became sensitive at higher concentrations of more than 800 μg/ml. Since the deregulation property of γ-glutamyl kinase, the key enzyme involved in the biosynthetic pathway of L-proline, could be used effectively for the overproduction of L-proline, selection of mutant strains resistant to proline analogs was considered to be crucial. They were considered to be free of feedback regulation by L-proline. The sensitivity against proline analogs was determined by cultivating the bacterial strains on a minimal medium supplemented with various concentrations of DHP, AZC, and TAC. The growth of four parent strains was

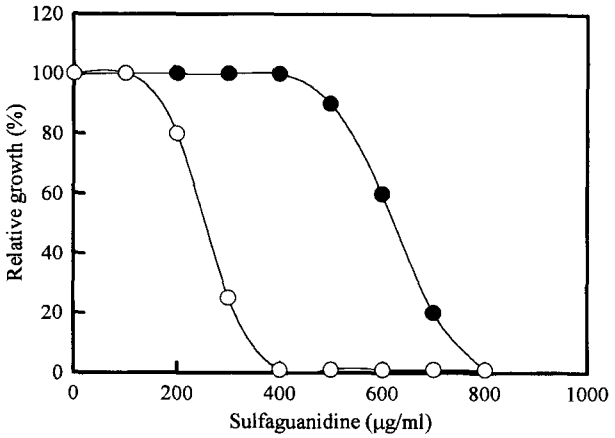

Fig. 1. Growth inhibition of C. *acetoacidophilum* RYU3161 (●) and parent strain, *C. acetoacidophilum* ATCC13870 (○), by sulfaguanidine.

completely inhibited by 8 mM DHP, 10 mM AZC, and 12 mM TAC. On the other hand, the mutant strain RYU3161 was almost insensitive at those concentrations of the analogs.

Isolation of L-Proline Overproducing Mutants

The cells were treated with NTG or UV irradiation according to the methods of Carlton [6] and Miller [13], and then screened on a shake-flask scale. A large number of L-isoleucine and L-histidine auxotrophic mutant strains which are also resistant to SG were derived from four L-glutamic acid-producing bacteria; *B. lactofermentum* ATCC 13869, *C. acetoacidophilum* ATCC 13870, *B. flavum* ATCC 15940, and *C. glutamicum* ATCC 15942 (Table 2).

L-Histidine auxotrophic and SG resistant strains derived from *C. acetoacidophilum* ATCC 13870 accumulated the highest level of L-proline (25 g/l). This mutant strain derived from *C. acetoacidophilum* ATCC 13870 was designated as the RYU strain. To remove feedback regulation by L-proline, the DHP, AZC, and TAC resistant mutant strains were then derived from these RYU strains by using serial mutagenesis treatments. In particular, since osmotic stress reportedly resulted in an increase of the L-proline biosynthesis rate [19, 20], proline analog-resistant mutants were screened under high osmotic pressure. After an extensive selection was made out of thousands of mutant strains, seven L-proline overproducing strains were isolated (Table 3).

Among these strains, the RYU3161 (KCTC 0616BP) strain produced the highest amount of L-proline (33 g/l), and was finally selected as the L-proline production strain for the purpose of conducting further fermentation studies. The activity of γ-glutamyl kinase of the mutant strain

Table 2. Isolation of L-proline-producing L-isoleucine and L-histidine auxotrophs resistant to sulfaguanidine from L-glutamic acid-producing bacteria.

Microorganism	No. of colonies tested	No. of producers isolated	Max. amount of L-proline (g/l)	Characteristics of best producing mutant*
Brevibacterium lactofermentum ATCC13869	415	41	19	Ileu$^-$, SG^r
Corynebacterium acetoacidophilum ATCC13870	477	57	25	His$^-$, SG^r
Brevibacterium flavum ATCC15940	180	21	11	Ileu$^-$, SG^r
Corynebacterium glutamicum ATCC15942	425	49	20	His$^-$, SG^r

*SG^r: sulfaguanidine resistant (400 μg/ml).

Table 3. Production of L-proline in a shake-flask culture by a proline analog-resistant mutant from the L-histidine auxotroph and sulfaguanidine resistant mutant of *C. acetoacidophilum* ATCC13870.

Strains	Dry cell wt. after 72 h cultivation (g/l)	Amount of L-proline accumulated (g/l)	Characteristics of best producing mutant*
RYU2102	12.3	28	DHP^r
RYU2204	12.5	28	DHP^r, AZC^r
RYU2703	12.1	30	DHP^r, AZC^r
RYU2810	12.2	29	DHP^r, AZC^r
RYU2955	12.5	31	DHP^r, AZC^r, TAC^r
RYU3001	12.4	32	DHP^r, AZC^r, TAC^r
RYU3161	12.2	33	DHP^r, TAC^r, TAC^r

*DHP^r: DL-3,4-dehydroproline resistant (10 mM).
AZC^r: Azetidine-2-carboxylic acid resistant (12 mM).
TAC^r: L-Thiazolidine-4-carboxylic acid resistant (12 mM).

Table 4. Activity of γ-glutamyl kinase in mutant strain RYU3161 and parent strain, *C. acetoacidophilum* ATCC13870, in YPD and fermentation media.

Strains	YPD medium		Fermentation medium	
	ATCC13870	RYU3161	ATCC13870	RYU3161
Total protein (g/l)	3.3	2.8	2.65	2.03
Enzyme activity (M)	0.48	0.60	0.59	0.74
Specific activity (μmol/g protein)	0.72	1.07	1.2	1.75
Unit (μmol/g protein/min)	3.64	5.45	6.17	10.1

Table 5. Fermentation parameters and L-proline production depending on different concentrations of ammonium sulfate.

Concentration of ammonium sulfate (%)	Specific growth rate (μ, hr^{-1})	Maximum cell mass (Xm, g/l)	Product yield ($Y_{p/s}$, g L-pro./g glu.)	Maximum product concentration (Pm, g/l)
1	0.243	20.1	0.071	7.1
3	0.206	12.7	0.33	33
5	0.131	8.9	0.23	24

RYU3161 was found to be 45% higher than that of the wild-type strain in both YPD medium and the fermentation medium (Table 4).

The biosynthetic pathways of L-proline and L-histidine are quite different according to the published metabolic studies [16]. Therefore, L-proline production by this L-histidine auxotrophic mutant RYU3161 is likely to indicate some interrelations existing between the L-proline and L-histidine pathways. Studies on both the L-proline productivity of RYU 3161 and an analysis of biochemical relations between L-proline and L-histidine biosynthesis are worthy of further investigation.

Media Optimization for Batch Fermentation

Since L-proline was synthesized from L-glutamic acid with energy supplement (in the form of ATP), hydrogen donors and amino groups, it required a large amount of nitrogen and an aerobic culture condition. Among inorganic nitrogen sources tested, ammonium sulfate was found to produce the highest titre of L-proline. However, an excess amount of ammonium sulfate not only inhibited the cell growth but also L-proline production. In contrast, a shortage of ammonium ion enhanced the formation of α-ketoglutaric acid, which resulted in a significant decrease in the yield of L-proline [1, 12]. The optimization of ammonium sulfate concentration was therefore essential for the overproduction of L-proline. *C. acetoacidophilum* RYU3161 was thus cultivated in a jar fermenter with various ammonium sulfate concentrations (Table 5).

It was noted that L-proline production was inhibited by concentrations of ammonium sulfate higher than 3%. The carbon and inorganic salt concentrations were also optimized by a series of experiments. It was observed that glucose was the most suitable carbon source, and cations such as K^+, Mg^{2+}, Fe^{2+}, and Mn^{2+}, as well as anions such as PO_4^{-3}, Cl- and SO_4^{-2} were essential for L-proline production. The optimal concentration of these inorganic salts were KH_2PO_4 0.3%, $FeSO_47H_2O$ 0.003%, $MnSO_44H_2O$ 0.003%, and $ZnSO_47H_2O$ 0.002%. On the contrary to the fact that biotin was known to be one of the most important factors for L-glutamic acid fermentation, it showed no significant effect on the production of L-proline. In addition, neither thiamine hydrochloride nor penicillin showed any significant effect on the production of this amino acid.

Effects of L-Histidine and Yeast Extract

The effects of various concentrations of L-histidine and yeast extract on L-proline production were studied with the mutant strain RYU3161 in shake-flask experiments (Fig. 2 and Table 6).

The optimal concentrations of L-histidine and yeast extract were found to be 0.16 g/l and 7 g/l, respectively. The mutant strain RYU3161 required L-histidine for growth, but, in excess concentration, the amino acid yield decreased.

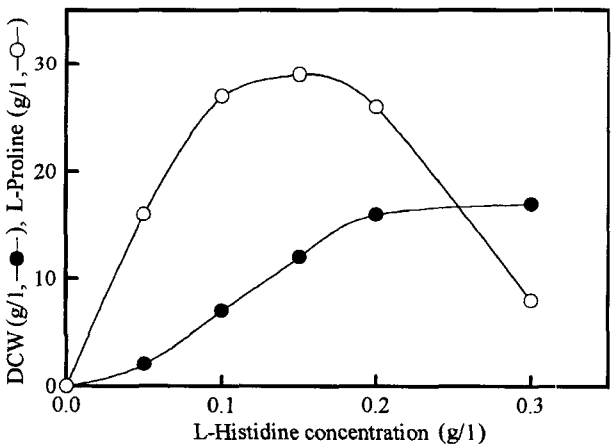

Fig. 2. Effect of L-histidine concentration on L-proline production and cell growth.

Table 6. Fermentation parameters and L-proline production depending on different concentrations of the yeast extract.

Concentration of yeast extract (%)	Specific growth rate (μ, hr^{-1})	Maximum cell mass (Xm, g/l)	Product yield ($Y_{p/s}$, g L-pro./g glu.)	Maximum product concentration (Pm, g/l)
0.5	0.128	9.91	0.27	27
0.7	0.175	12.6	0.33	33
0.9	0.190	15.3	0.31	32
1.2	0.210	18.2	0.23	23
1.6	0.231	21.2	0.18	18

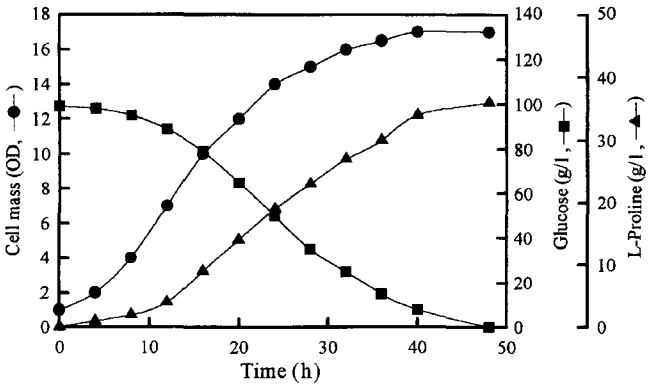

Fig. 3. Time profile of the cell mass, and glucose and L-proline concentrations during the batch culture of *C. acetoacidophilum* RYU3161.

Since γ-glutamyl kinase is not directly regulated by L-histidine [4, 28, 29], it is believed that certain amino acid metabolisms in this strain are strongly blocked by an excess amount of L-histidine, enhancing the supply of ATP and carbon flux to increase the rate of L-proline biosynthesis. Further study is therefore needed to elucidate more clearly a metabolic shift in this microorganism.

Batch Fermentation of L-Proline

Batch fermentation experiments have been conducted in a 5-l jar fermenter containing 2 l of the culture medium. Ammonium sulfate at a concentration level of 30 g/l was used as an inorganic nitrogen source and 7 g/l of yeast extract as an organic nitrogen source. Since yeast extract contained about 2.4% L-histidine, this level of yeast extract could supply L-histidine at a concentration of 0.16 g/l. Cell mass and L-proline concentration profiles during the batch fermentation are shown in Fig. 3. The final concentration of L-proline was 35 g/l and its production was growth associated. It is of interest to point out that the concentrations of other amino acids were very small.

Conclusions

Various mutant strains were derived from L-glutamic acid-producing bacteria by using conventional mutation techniques. Among these mutant strains, *Corynebacterium acetoacidophilum* RYU3161 was successfully isolated. The activity of γ-glutamyl kinase of the mutant strain was 45% higher than that of the parent strain. The fermentation condition for batch production of L-proline was optimized and it was found that a feeding of 0.16 g/l of L-histidine was necessary to enhance L-proline production. As a result, 35 g/l of L-proline was produced with a molar yield of 54% within 48 h of batch fermentation, which was considered reasonably appropriate for an industrial application.

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