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Improvement of Culture Conditions for L-Proline Production by a Recombinant Strain of *Serratia marcescens*

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ABSTRACT

Serratia marcescens SP511 was previously reported to be an L-proline-producing strain that harbors a recombinant plasmid carrying the mutant type of the proline operon. This strain produced 65 g/L of L-proline in a medium containing 22% sucrose and urea after 5 d of incubation under the conventional culture conditions. We searched for more suitable culture conditions for more abundant L-proline production by SP511. To improve the supply of a nitrogen source to cells, ammonium was used instead of urea and fed to a culture under control of the pH of the medium. The concentrations of $MgSO_4$ and K_2HPO_4 were increased, and in addition, sucrose was continuously added to the culture at a final concentration of 32%. Under these conditions, the cell amount was increased twofold over that under the previous conditions and L-proline production reached a maximum of more than 100 g/L after 4 d of incubation.

Index Entries: L-proline production; nitrogen source; ammonia; *Serratia marcescens*.

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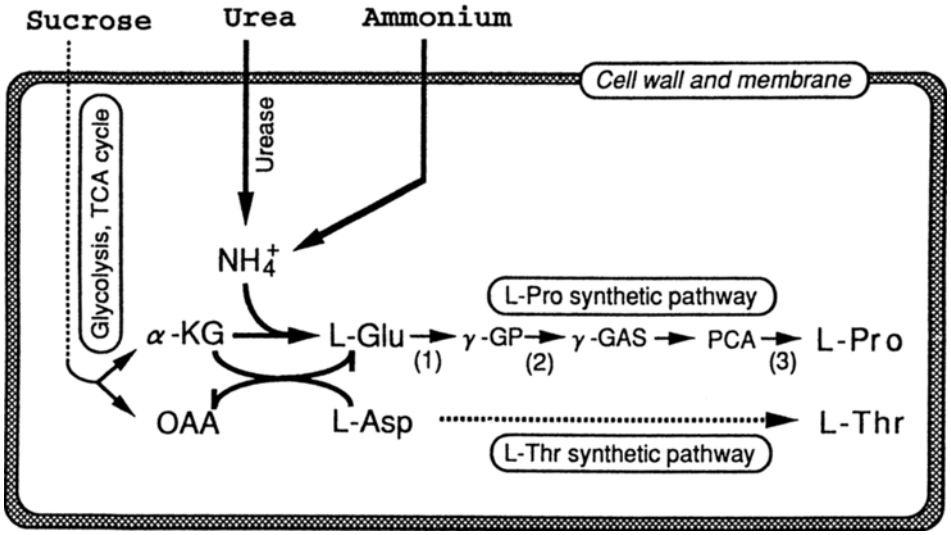

Fig. 1. Pathway of L-proline and L-threonine biosynthesis in *S. marcescens*. Abbreviations: α-KG, α-ketoglutarate; OAA, oxaloacetate; L-Glu, L-glutamate; L-Pro, L-proline; L-Thr, L-threonine; γ-GP, γ-glutamyl phosphate; γ-GSA, γ-glutamyl semialdehyde; PCA, pyrroline-5-carboxylate; (1), γ-glutamyl kinase; (2), γ-glutamyl phosphate reductase; (3), 1-pyrroline 5-carboxylate reductase.

INTRODUCTION

L-Proline is used mainly as one of the raw materials of amino acid infusion agents and as a food additive (*1*). This amino acid has been commercially produced through fermentation methods (*1*), because chemical synthetic methods cannot provide L-proline with a high quality at a low cost. The recent increasing demand for L-proline needs the development of more economical methods.

L-Proline is synthesized from L-glutamic acid via three enzymatic reactions in microorganisms (Fig. 1) (*2*). The rate-limiting enzyme γ-glutamyl kinase (*proB* product) is subject to proline-mediated feedback inhibition. Feedback repression does not function on the proline biosynthetic enzymes, including the second enzyme, γ-glutamyl phosphate reductase (*proA* product), and the last enzyme, 1-pyrroline 5-carboxylate reductase (*3–5*). We have been constructing various L-amino acid-producing strains using *Serratia marcescens* (*6,7*). *S. marcescens* SP187 (*8*) is an L-proline-producing strain, derived from a wild strain by four rounds of mutagenesis. This mutant has three mutations involved in the resistance to proline analogs and, in addition, one mutation related to defectiveness of proline degradation. Recently, Omori et al. have reported that, in SP187, γ-glutamyl kinase was insensitive to feedback inhibition and the *proBA* genes were overexpressed (*5,9*). SP187 produced 55 g/L of L-proline in a medium containing sucrose and urea. The *proBA* genes were

cloned from the chromosomal DNA of SP187 onto a mini-F plasmid vector, yielding a recombinant plasmid, pYI350 (*10*). Strain SP511, obtained by introducing pYI350 into SP187, was found to produce L-proline at 65 g/L under the conventional culture conditions (*10*). This productivity was lower than that expected on the basis of the gene dosage effect.

We previously reported an L-threonine-producing strain of *S. marcescens*, which was constructed by cloning the mutant type of the threonine operon (*6*) and introducing a resultant recombinant plasmid into an L-threonine-producing mutant (*6*). The productivity of this recombinant strain was unexpectedly low under the previous culture conditions, but increased by improving the supply of a nitrogen source and feeding a carbon source (*11*). This study has indicated that a maximum production by a recombinant strain required specific culture conditions different from those for a nonrecombinant strain. Here, we report more optimum culture conditions for more abundant L-proline production by a recombinant strain, *S. marcescens* SP511.

MATERIALS AND METHODS

Microorganisms and Media

S. marcescens SP511 (*10*), a recombinant L-proline-producing strain, was used for this study. *S. marcescens* SP187 (*8*), which was used as the host of SP511, was an L-proline-producing strain with multiple mutations releasing feedback controls. SP511 harbored a recombinant plasmid, pYI350 (*10*), which carried the mutant-type *proBA* genes. A seed medium contained 10% sucrose, 1.5% urea, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0002% $FeSO_4 \cdot 7H_2O$, 1.5% corn steep liquor (CSL), 2% sodium L-aspartate monohydrate, 3% $CaCO_3$, and 0.01% kanamycin sulfate (Km). The urea medium for the batch cultures contained 22% sucrose, 2.8% urea, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0002% $FeSO_4 \cdot 7H_2O$, 0.6% CSL, 0.1% yeast extract, 3% $CaCO_3$, 0.2% antifoamer DISFOAM CC438 (Nippon Oils and Fats Co., Ltd.), and 0.01% Km. The ammonia medium of the batch culture consisted of the same composition as that of the urea medium, except that urea and $CaCO_3$ were not added. The starting medium of the fed-batch cultures contained 26% sucrose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0002% $FeSO_4 \cdot 7H_2O$, 0.6% CSL, 0.1% yeast extract, 0.27% antifoamer, and 0.01% Km. The feed medium contained 58% sucrose, 0.0002% $FeSO_4 \cdot 7H_2O$, 0.6% CSL, and 0.1% yeast extract.

Culture Methods

Seed cultures for fermentations were started by inoculating two loopfuls of cells grown at 30°C for 20 h on an LB (Luria broth) agar slants supplemented with 0.01% Km to 500-mL flasks containing 30 mL of seed culture medium. Flasks were incubated at 30°C for 24 h at 140 rpm on a

reciprocal shaker. Twenty-four milliliters of seed cultures were inoculated into the jar fermentor. Batch cultures and fed-batch cultures were carried out using 2-L jar fermentors (Iwashiya, model MBW-2) containing 1200 mL of the medium and 2-L jar fermentors containing 900 mL of the starting medium, respectively. The feeding was initiated at 47 h after inoculation and continued until 91 h at a constant rate of 6.8 mL/h. In addition, 2 mL of 20% $MgSO_4 \cdot 7H_2O$ and 2 mL of 20% K_2HPO_4 were added at 32, 47, and 71 h. When cultured in the ammonia medium, 28% ammonia water was fed to the culture, controlling the pH of culture medium. The pH was continuously monitored using a sterilizable pH electrode (INGOLD, model DPAS/120) attached to a pH controller (Tokyo Rikakikai Co., Ltd., model FC-1) with setpoint limit of pH 5.6. Supply of ammonia depended on on–off activation of a peristaltic pump (Tokyo Rikakikai Co., Ltd., model MP-3). The concentration of dissolved oxygen was maintained above 2 ppm by changing the rate of agitation. The culture temperature was maintained at 30°C.

Analytical Methods

Biomass was determined spectrophotometrically at 660 nm and expressed as dry wt/L calculated by comparison with a standard conversion curve. L-Proline was measured by bioassay with *Leuconostoc mesenteroides* P-60. Sucrose and urea were measured by the method of Dubois et al. (*12*) and the diacetyl monoxim method (*13*), respectively. Ammonium ion was measured with an ammonia gas electrode (Denki Kagaku Keiki, model 7161).

RESULTS

Typical Changes during L-Proline Production Using Urea as a Nitrogen Source

First, we examined typical changes during L-proline production by *S. marcescens* SP511 in the medium containing urea as a nitrogen source. When urea was used as a nitrogen source, the cell growth was accompanied by the increase of L-proline production (Fig. 2A). Sucrose and urea were gradually consumed as the growth proceeded. L-Proline production reached a maximum of 65 g/L, and sucrose was barely detectable at 5 d. The concentration of ammonium, which might be formed from urea through sterilization of medium, was 0.5 g/L at the beginning of the incubation, gradually decreased as cells grew, and was barely detectable at 1 d. Ammonium was detected again at 2 d, and the concentrations were maintained at 0.1–0.2 g/L during the incubation. The above results indicated at the ammonium was in short supply for L-proline production with the urea medium until 2 d, but after 2 d, not in short supply.

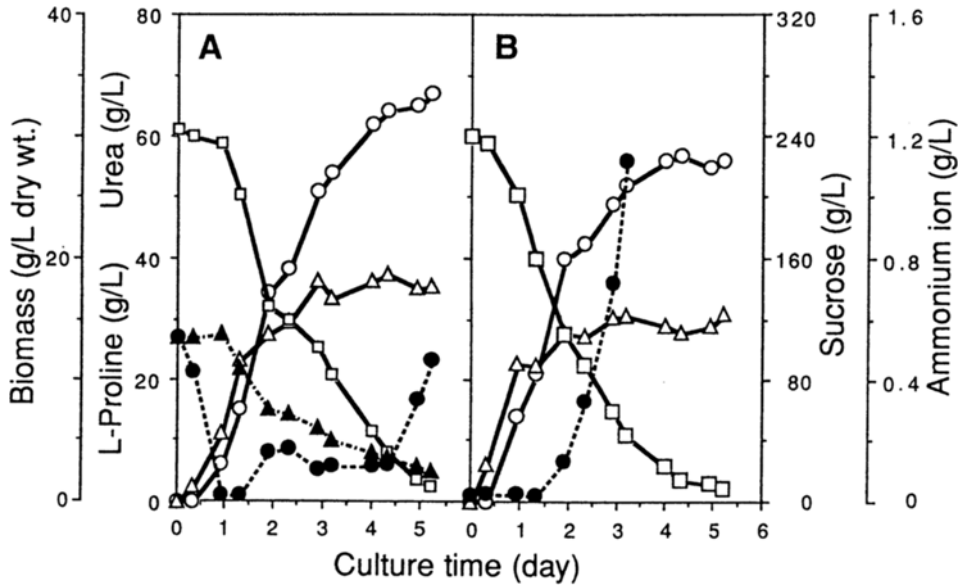

Fig. 2. Time-course of L-proline production by *S. marcescens* SP511 using urea (A) or ammonium (B) as a nitrogen source. Growth (△); L-proline produced (○); sucrose (□); urea (▲); ammonium ion (●).

L-Proline Production in the Medium to Which Ammonium Was Fed as a Nitrogen Source

We intended to increase the L-proline production by finding more suitable culture conditions, including feeding a nitrogen and a carbon source. For this purpose, ammonium was used as a nitrogen source instead of urea, because the former nitrogen source was expected to be more readily assimilated by cells than the latter (Fig. 2B). Then ammonium water was fed into the culture so as to keep the pH of the medium below 6.5. The concentrations of ammonium were at a concentration as low as 0.02 g/L until 1 d, increased rapidly at 2 d, and reached over 1 g/L at 3 d. Cells grew faster under these conditions than under previous conditions, accompanied by faster L-proline production, but the final cell and L-proline amounts were reduced as compared with those obtained for the medium containing urea.

Improvement of the Medium Composition for Accelerating the Ammonium Assimilation

We examined the composition of medium in order to accelerate the ammonium assimilation and to decrease the ammonium concentrations in the culture with the ammonium medium. Because ammonium is assimilated via the reaction with α-ketoglutarate (α-KG), ammonium assimilation is expected to be accelerated by increasing of α-KG supply. Further, it is

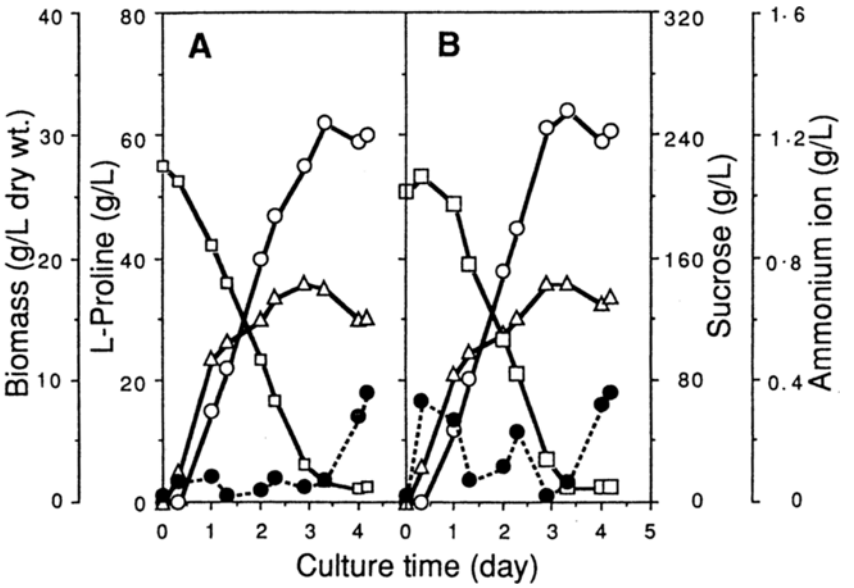

Fig. 3. Effects of increasing of CSL or $MgSO_4 \cdot 7H_2O$ on L-proline production using ammonium as a nitrogen source. A: The concentration of CSL was increased from 0.6 to 0.9%. B: The concentration of $MgSO_4 \cdot 7H_2O$ was increased from 0.05 to 0.2%. Growth (△); L-proline produced (○); sucrose (□); ammonium ion (●).

effective for increasing α-KG supply to accelerate glycolysis because α-KG is formed through glycolysis. Magnesium is a cofactor for several enzymes of glycolysis, and CSL includes growth factors, such as vitamins, nucleic acid, and trace elements. Hence, we investigated the effects of concentrations of $MgSO_4 \cdot 7H_2O$ and CSL on the assimilation of ammonium in cultivation of SP511. When the concentrations of CSL and $MgSO_4 \cdot 7H_2O$ were increased from 0.6 to 0.9% and from 0.05 to 0.1%, respectively, the ammonium concentrations were kept at 0.1–0.4 g/L during the entire period of incubation (Fig. 3A,B). When the concentrations of CSL and $MgSO_4 \cdot 7H_2O$ were increased, the maximum proline production was 65 mg/L at 3 d. This value was almost the same as that observed for the urea medium, but the cultivation time was shortened by 2 d.

Increasing L-Proline Production by Feeding of Sucrose Using Ammonium as a Nitrogen Source

The L-proline production was expected to be increased by feeding additional sucrose to the medium containing higher concentrations of CSL and $MgSO_4 \cdot 7H_2O$ when ammonium was used as a nitrogen source. Fed-batch cultures were hence studied as follows. Ammonia water was supplied as described above, and in addition, the feed medium contain-

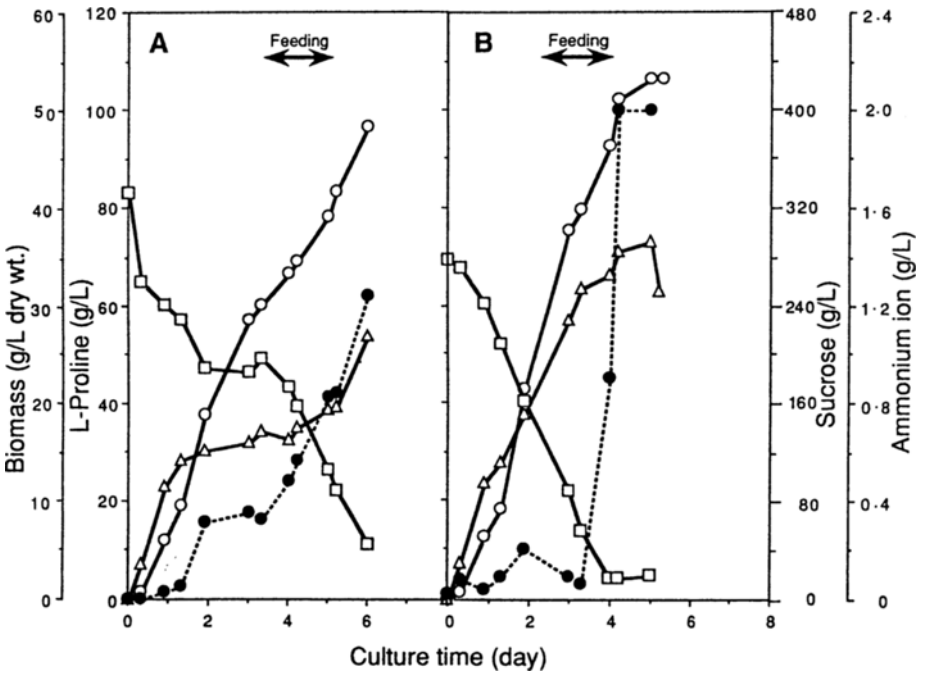

Fig. 4. Fed-batch culture for L-proline production by *S. marcescens* SP511 using ammonium as a nitrogen source. A: The concentration of $MgSO_4 \cdot 7H_2O$ was increased from 0.5 to 0.15%. B: The concentrations of $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 were increased from 0.05 to 0.15% and from 0.1 to 0.2%, respectively. Feeding: sucrose and other nutrients were continuously added. Growth (△); L-proline produced (○); sucrose (□); ammonium ion (●).

ing sucrose and other nutrients was continuously added to the fermentor during the period of 47–91 h (Fig. 4A). The concentration of $MgSO_4 \cdot 7H_2O$ was 0.05% at the beginning of cultivation, and 2 mL of 20% $MgSO_4 \cdot 7H_2O$ solution were added to the medium at 32, 47, and 71 h. As a result, the final concentration of $MgSO_4 \cdot 7H_2O$ reached 0.15%. The ammonium concentrations were kept below 0.4 g/L until 3 d, but increased sharply to 1.2 g/L at the end of cultivation. Therefore, we examined the composition of medium again. When the concentrations of both $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 were increased, ammonium concentrations were kept at < 0.2 g/L through the cultivation (Fig. 4B). The growth reached a maximum after 4 d of cultivation, and the cell mass was increased to 36 g/L from 18 g/L. The latter value was obtained for conventional batch cultures using urea as a nitrogen source. As a result, the maximum L-proline was reached at about 100 g/L or more. The total amount of sucrose consumed was 340 g/L. Thus, we established an improved culture method for L-proline production by *S. marcescens* SP511.

DISCUSSION

Generally, the high yield of products through fermentative methods requires optimum culture conditions, in particular, efficient supply of carbon and nitrogen sources to culture. In the microbial production of L-amino acids, ammonium sulfate and ammonia water have been frequently used as nitrogen sources. Because high concentrations of ammonium inhibit cell growth and L-amino acid synthesis, various methods have been studied for maintaining ammonium at low concentrations during the entire period of fermentation (*14–16*). Nevertheless, it is probably difficult to use most of them for industrial process because of complicated operation. On the other hand, we have been using urea as a nitrogen source for L-amino acid production by *S. marcescens* strains (*6,7*) because this bacterium is able to assimilate urea, which has a lesser effect on the growth even at high concentrations.

In L-threonine production with a recombinant strain of *S. marcescens*, the productivity was lower than that expected on the basis of gene dosage effect under the conventional culture conditions (*11*). We found that one of the reasons for low productivity was the shortage of ammonium supply from urea. SP511, which was constructed from the same wild strain as that of a recombinant L-threonine producer, was also unable to express the potential productivity, based on the gene dosage, under the conventional culture conditions. A key precursor of L-proline is L-glutamic acid, and therefore, the rate of L-proline synthesis primarily depends on that of L-glutamic acid formation. More abundant production of L-proline requires the increase of formation of L-glutamic acid, which is synthesized from α-KG and ammonium via amination by L-glutamic dehydrogenase. In this study, we supposed that the formation of ammonium from urea and α-KG from sugar might be, in short, for increasing the L-proline production under previous culture conditions. For increasing ammonium supply, ammonia water was fed while monitoring the pH of the medium. Growth rate in the early phase increased, but the final cell mass and L-proline production were decreased compared with the culture using urea medium. Then we accelerated the anabolic metabolism of the carbon source by increasing concentrations of K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and CSL. This altered medium composition provided cells with more rapid growth and enabled us to feed additional amounts of sucrose to culture, resulting in more abundant production of L-proline and shortened the period of the incubation to reach a maximum production (Table 1).

Recently, recombinant strains for L-amino acid production have been constructed. In many recombinant strains, activities of the key enzymes were increased by the gene engineering. However, the shortage of precursor supply frequently prevents recombiant strains from expressing the potential productivity based on the gene dosage. For optimization of culture conditions, it is important to analyze the time-course of cultivation

Table 1
Summary of L-proline Production under Various Conditions

Nitrogen source	Sucrose, %	Further addition[a]	Culture time, d	Maximum of L-proline production, g/L
Urea	22	None	5	65
Ammonium	22	None	4	55
Ammonium	22	$MgSO_4$	3	65
Ammonium	34[b]	$MgSO_4$, K_2HPO_4	4	100

[a]During incubation.
[b]The final concentration after feeding.

in detail: the increase of cells and products, the consumption of carbon and nitrogen sources, and changes of another components in the culture medium. Moreover, it is necessary to find what is lacking in cell metabolisms and to supply it effectively to the culture.

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