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Improvement of Nitrogen Supply for L-Threonine Production by a Recombinant Strain of *Serratia marcescens*

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ABSTRACT

Serratia marcescens T-2000 was previously reported to be an L-threonine-producing strain that harbors the recombinant plasmid carrying the mutant-type threonine operon. This strain produced 55 g of L-threonine/L of the medium containing urea as a nitrogen source after 72 h of cultivation. In the urea-containing medium, transitory stop of the growth was observed during the early period of cultivation when the entire amount of ammonium ion formed from urea via heat decomposition disappeared in the medium. This indicated that the shortage of ammonium supply in cells might delay both the cell growth and the L-threonine production. The use of ammonia water as a nitrogen source for L-threonine production was therefore studied, because microbial cells generally assimilate this source more readily than urea. When ammonia water was automatically fed to the medium so as to maintain the pH of the medium at around 7, the growth was accelerated, and the L-threonine production reached a maximum of 65 g/L at 48 h. Under these conditions, sucrose, a carbon source, was continuously fed to the medium, resulting in the production of 100 g of L-threonine/L at 96 h. Thus, the L-threonine productivity of the recombinant L-threonine-producing strain could be increased by devising the method for supply of a nitrogen source.

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

INTRODUCTION

L-Threonine has been mainly used for medical purposes, such as infusion agents. Recently, this essential amino acid has been given attention as feeding supplements for domestic animals to improve feed efficiency. More economical production should be developed for the feed market. There are four stereoisomers for threonine; D, L, D-allo, and L-allo forms. Synthetic chemical methods are therefore incapable of providing the L-forms with a high purity in a high yield. Accordingly, the microbial process is of greater advantage than the chemical synthesis for the industrial production of L-threonine.

Serratia marcescens is one of the convenient strains for amino acid production. *S. marcescens* strains producing L-threonine were constructed through transductional and recombinant methods (*1–3*). Aspartokinase and homoserine dehydrogenase are key enzymes of threonine synthesis. Seven regulatory mutations for three aspartokinases and two homoserine dehydrogenases were combined in a single strain defective in two threonine-degrading enzymes through transductional crosses, yielding a threonine-producing strain, T-1165 (*thrA$_1$5A$_2$5, hnrA1B2 lysC1, aec-1, etr-21*). T-1165 produced 40 g of L-threonine/L of the medium containing sucrose and urea. The mutant-type threonine operon (*thrA$_1$5A$_2$5BC*) was cloned from the chromosomal DNA of this strain onto a low-copy-number vector pLG339 (*4*), a derivative of pSC101, yielding pSK301. This recombinant plasmid was introduced into strain T-1165, yielding a recombinant strain, T-2000 (*5*). Under the conventional cultural conditions, this strain produced L-threonine at only 50–60 g/L. This productivity was lower than the expected value based on the copy number of the plasmid used. There was a possibility that the cultural conditions might be inadequate for allowing T-2000 to express its potential for the maximum production. This work improved the method for the supply of a nitrogen source to increase the L-threonine productivity of T-2000.

MATERIALS AND METHODS

Microorganisms and Media

L-Threonine-producing strains, *S. marcescens* T-1165 (*1*) and T-2000 (*5*), were used in this study. T-2000 harbors recombinant plasmid pSK301, which carries the aspartokinase I (AKI) gene and homoserine dehydrogenase I (HDI) gene. The urea medium for the batch cultures contained 15% sucrose, 1.5% urea, 0.05% $(NH_4)SO_4$, 0.1%, K_2HPO_4, 0.1%

$MgSO_4 \cdot 7H_2O$, 0.0002% $FeSO_4 \cdot 7H_20$, 0.1% corn steep liquor, 0.1% yeast extract, 0.043% L-isoleucine, 0.049% L-methionine, 2% $CaCO_3$, and 0.5% antifoamer COLORIN 202 (Sanyo Chemical Industries, Ltd.). The ammonium medium had the same composition as that of the urea medium, except that urea was not involved. The starting medium for the fed-batch cultures contained 10% sucrose, 0.05% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0002% $FeSO_4 \cdot 7H_20$, 0.1% corn steep liquor, 0.1% yeast extract, 0.043% L-isoleucine, 0.049% L-methionine, 2% $CaCO_3$, and 0.5% antifoamer COLORIN 202. The feed medium contained 65% sucrose, 0.05% $(NH_4)SO_4$, 0.34%, K_2HPO_4, 0.0002% $FeSO_4 \cdot 7H_20$, 0.1% corn steep liquor, 0.34% yeast extract, 0.043% L-isoleucine, and 0.049% L-methionine.

Culture Methods

The batch cultures were performed in a 2-L jar fermentor containing 1200 mL of the medium. The fed-batch cultures were performed in a 2-L jar fermentor containing 700 mL of the starting medium. The feeding was initiated at 25 h after inoculation and continued to 80 h at a constant rate of 7.3 mL/h. In addition, 4.8 mL of 20% $MgSO_4 \cdot 7H_2O$ were added at 55 h. When cultured in the ammonium medium, 28% ammonia water was fed to the culture, controlling the pH of the culture medium. The pH was continuously monitored using a sterilizable pH electrode (INGOLD model DPAS/120) attached to pH controller (Tokyo Rikakikai Co., Ltd. model FC-1) with setpoint limits of pH 7.2. 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 27°C.

Analytical Methods

Biomass was determined spectrophotometrically at 660 nm, and turbidity was converted to dry wt/L (g/L dry wt) by comparison with a standard conversion curve. L-Threonine production was measured by bioassay with *Leuconostoc mesenteroides* P-60. Sucrose was measured by the method of Dubois et al. (*6*). Urea was measured by the diacetyl monoxim method (*7*). Ammonium ion was measured with an ammonia gas electrode (Denki Kagaku Keiki model 7161).

Enzyme Assays

For the preparation of cell extracts, growing cells were harvested from the culture by centrifugation, disrupted with a sonic oscillator, and centrifuged as described previously (*8*). Glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) were determined by measuring the oxidation of NADPH by the method of Dendinger et al. (*9*). Glutamine synthetase (GS) was measured by a modification of the γ-glutamyltransferase

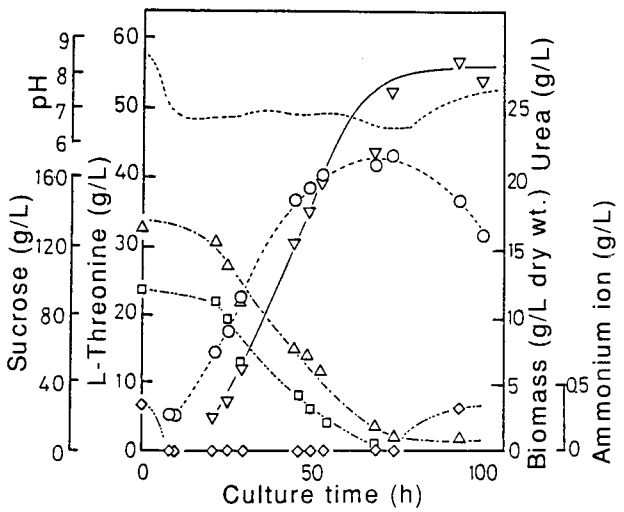


Fig. 1. Time-course of L-threonine production by *S. marcescens* T-2000 using urea as a nitrogen source (0–4 d). Growth (○); L-threonine produced (▽); sucrose (△); urea (□); ammonium ion concentration (◇); pH of medium (---).

assay (*10*). Urease was estimated by determining the quantity of urea decomposed at 30°C. The reaction mixture contained 1% urea in a total vol of 1 mL of 25 m*M* phosphate buffer (pH 7.0) and 3 m*M* ethylene diamine tetraacetate. The reaction was initiated by the addition of cell extracts and terminated by the addition of 0.5 mL of 15% perchloric acid. Protein was measured by the use of a Bio-Rad protein assay kit with bovine serum albumin as a standard protein.

RESULTS

Typical Changes During L-Threonine Production Using Urea as a Nitrogen Source

First, typical changes during L-threonine production by *S. marcescens* T-2000 in the medium containing urea as a nitrogen source were examined. When urea was used as a nitrogen source, the cell growth was accompanied by the increase of L-threonine production (Fig. 1). Sucrose and urea were slowly consumed and barely detectable when the growth was stopped. The maximum L-threonine production of 55 g/L was observed at about 75 h. The ammonium concentrations were very low, that is, < 0.001 g/L, during the logarithmic growth period. The growth and ammonium concentrations at the early growth phase were examined in detail (Fig. 2). After a lag period of 6 h, the rapid cell growth was observed, but at 10 h, the growth stopped for about 2 h, followed by restarting of the growth.

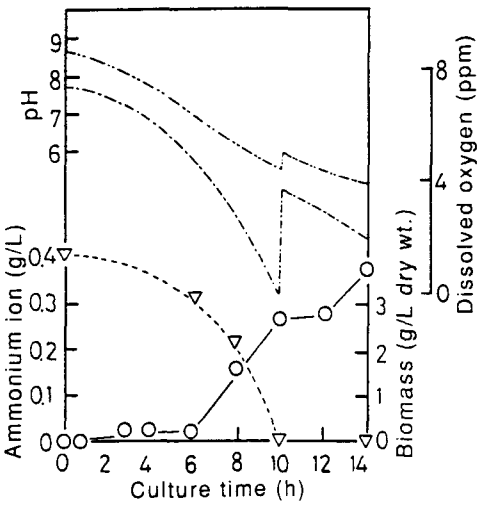


Fig. 2. Time-course of L-threonine production by *S. marcescens* T-2000 using urea as a nitrogen source (0–14 h). Growth (○); ammonium ion concentration (▽); dissoled oxygen concentration (·····); pH of medium (·-·-·-·).

Namely, a diauxie growth pattern was observed (*11*). At the beginning of the incubation, 0.4 g of ammonium ion/L was detected. This ammonium was supposed to be generated by the decomposition of urea during autoclave sterilization of medium. At 10 h, when the growth stopped, no ammonium was detected in the medium. Sudden increase of the dissolved oxygen concentration and the pH of medium were observed along with the stop of the growth. The process of the above diauxie growth was speculated as follows. At the early stage of the growth, cells assimilate ammonium in the medium as a first nitrogen source. After ammonium is exhausted from the medium, cells assimilate urea as a second nitrogen source through the catalysis by urease. The temporary cessation of growth seems to be the result of the induction of urease synthesis. There was a possibility that ammonium might be a better nitrogen source for cell growth than urea, because the former is more readily assimilated than the later.

Activities of Enzymes Related to Nitrogen Metabolism

The activities of enzymes related to nitrogen metabolism were measured with cell extracts from *S. marcescens* T-2000, which was grown in the medium containing urea (Table 1). Urease catalyzes the degradation of urea to ammonium and carbon dioxide. Glutamate dehydrogenase (GDH) catalyzes the formation of glutamate from ammonium and α-ketoglutarate when the ammonium concentration is high in the medium. Under

Table 1
Activities of Enzymes Related to Nitrogen Metabolism in *S. marcescens* T-2000 During L-threonine Production Using Urea as a Nitrogen Source

Culture time, h	NH_4^+, g/L	Sp act, nmol/min mg protein			
		Urease	GDH[a]	GOGAT[b]	GS[c]
7	0.250	5.4	40	92	19
23	<0.001	3.6	10	44	18
29	<0.001	4.4	14	47	19
48	<0.001	5.1	6	56	18
96	0.300	4.4	7	16	20

[a]GDH = glutamate dehydrogenase.
[b]GOGAT = glutamate synthase.
[c]GS = glutamine synthetase (assayed in the presence of Mg^{2+}, representing deadenylylated enzyme).

the conditions where ammonium is at low concentrations, glutamate synthase (GOGAT) and glutamine synthetase (GS) function as a system for the assimilation of ammonium.

Urease activities were monitored before and after the temporary cessation of the diauxie growth. Urease was synthesized constitutively: Unexpectedly the temporary cessation might not be owing to the induction of urease synthesis. GDH activities, which is one of ammonium assimilation enzyme, were high at 7 h before the temporary cessation, and thereafter decreased. However, even at 96 h, GDH activities remained. Concerning another ammonium assimilation enzymes, GS and GOGAT activities were detectable during the entire period of the cultivation.

L-Threonine Production in the Medium Containing Ammonium as a Nitrogen Source

The above results indicated that the slow supply of ammonium from urea might lead to delayed cell growth and L-threonine production. Hence, it was expected that the use of ammonia water instead of urea as a nitrogen source might accelerate the growth and L-threonine production, and then this possibility was tested (Fig. 3). Ammonia water was fed into the medium so as to keep the pH of the medium at 7.2. The concentrations of ammonium in the medium were 0.1–0.3 g/L during the entire period of cultivation. In this culture, most of sucrose added was consumed at 55 h, and the L-threonine production reached 65 g/L. The use of ammonium as a nitrogen sourse shortened the culture time required for the maximum production by about 1 d and increased by 10 g/L.

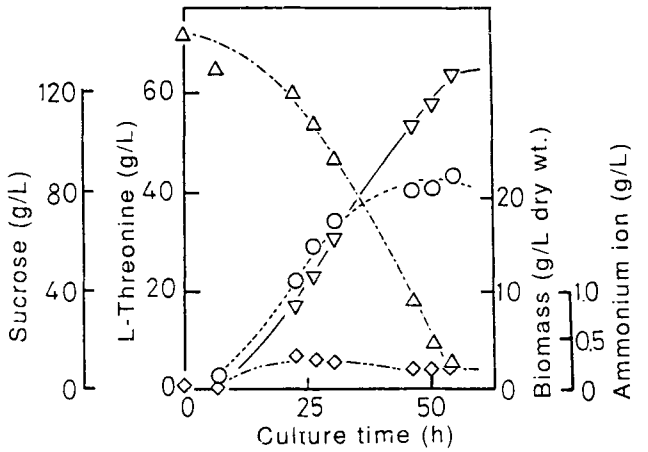


Fig. 3. L-Threonine production by *S. marcescens* T-2000 using ammonium as a nitrogen source. Growth (○); L-threonine produced (▽); sucrose (△); ammonium ion concentration (◇).

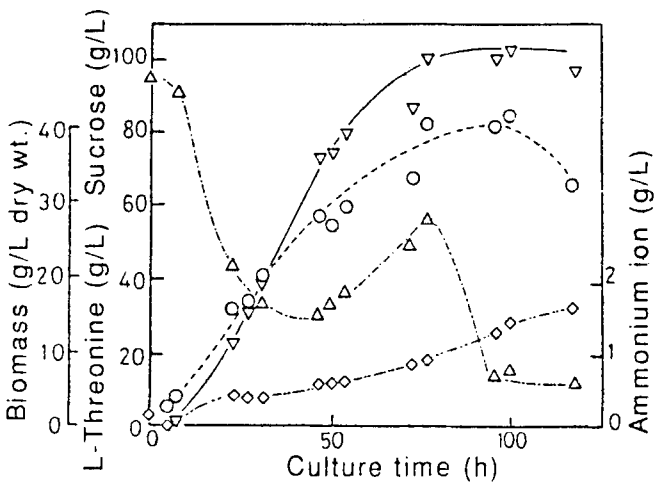


Fig. 4. Fed-batch culture for L-threonine production by *S. marcescens* T-2000 using ammonium as a nitrogen source. Growth (○); L-threonine produced (▽); sucrose (△); ammonium ion concentration (◇).

Fed-Batch Culture for L-Threonine Production Using Ammonium as a Nitrogen Source

Feeding of additional amount of sucrose was expected to increase the L-threonine production when ammonium was used as a nitrogen source. Hence, fed-batch culture was studied (Fig. 4). Ammonia water was supplied as described above, and in addition, the feed medium containing

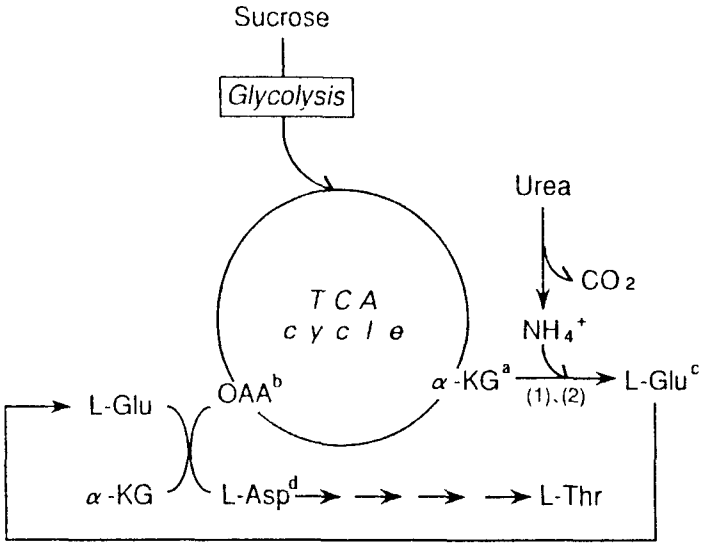
sucrose and other nutrients was continuously added to the fermentor during the period of 25–80 h. The concentrations of ammonium in the medium were 0.5–1.0 g/L during the entire period of cultivation. At 75 h, the growth reached the maximum, and L-threonine was produced at about 100 g/L. The total amount of sucrose consumed was 300 g/L. The cell mass was increased to about 40 g/L from 22 g/L (obtained for conventional cultures using urea as a nitrogen source). Thus, an improved culture method for L-threonine production by *S. marcescens* T-2000 was established.

DISCUSSION

Generally in fermentative production, the high productivity requires the optimization of supply of carbon and nitrogen sources to the cells. It is possible that inorganic nitrogen sources, such as ammonia water and ammonium salts, are used as cheap nitrogen sources for fermentative production. However, these inorganic compounds tend to inhibit the growth of cells and the metabolite production when the concentrations are increased. Hence, various control methods for ammonium concentration have been developed (*12–14*). However, probably it is difficult to apply these methods to industrial production. On the other hand, we have been using urea as a nitrogen source for L-threonine produciton, because *S. marcescens* has the ability of urea assimilation and the control of urea feeding is easier than that of ammonium feeding.

The pathway of L-threonine production by *S. marcescens* T-2000 was supposed to be the following process (Fig. 5). Sucrose is metabolized to α-ketoglutarate (α-KG) through glycolysis and TCA cycle. Urea is decomposed to ammonium by the catalysis of urease. L-Glutamate is synthesized from α-KG and ammonium. Subsequently, L-glutamate and oxaloacetate serve as substrates being converted to α-KG and L-aspartate. L-Aspartate is converted to L-threonine through the L-threonine biosynthetic pathway. There are two main pathways of L-glutamate synthesis. In the case of *E. coli*, at low ammonium concentrations, typically at <0.1 m*M*, L-glutamate is aminated by glutamine synthetase to form L-glutamine (*15*). High ammonium concentrations inhibit the synthesis of glutamine synthetase and stimulate the synthesis of L-glutamate by GDH. The synthesis of L-glutamate by GS and GOGAT requires ATP as an energy source. Therefore, the glutamate synthesis by GDH is an advantageous route for industrial fermentation production as compared with the synthesis by GS and GOGAT.

Accordingly, in industrial fermentation, it is important that ammonium concentrations be maintained above 0.1 m*M* (0.0018 g/L). This article has revealed that there were two pathways of L-glutamate synthesis in *S. marcescens* T-2000. However, it was supposed that L-glutamate was synthesized via the disadvantageous route, the GS-GOGAT pathway, because



(1) α-KG + NH_3 + NADPH $\xrightarrow{GDH^e}$ L-Glu + $NADP^+$

(2) L-Glu + NH_3 + ATP $\xrightarrow{GS^f}$ L-Gln + ADP + Pi

L-Gln + α-KG + NADPH $\xrightarrow{GOGAT^g}$ 2 L-Glu + $NADP^+$

Fig. 5. Pathway of L-threonine biosynthesis in *S. marcescens*. α-KG^a = α-ketoglutarate, OAA^b = oxaloacetate, L-Glu^c = L-glutamate, L-Asp^d = L-aspartate, GDH^e = glutamate dehydrogenase, $GOGAT^f$ = glutamate synthase, GS^g = glutamine synthetase.

ammonium concentrations were <0.1 m*M* during cultivation using the urea medium. Therefore, it is considered that urea is not a suitable nitrogen source for L-threonine production. Hence, ammonium was supplied by controlling the pH of the medium with 28% ammonia water. The efficient supply of ammonium resulted in increasing L-threonine production and shortening culture time. L-Threonine production by *Brevibacterium lactofermentum* (*16*) and *E. coli* (*17*) has been reported. Those L-threonine producers accumulated 33–75 g/L of L-threonine. In this work, *S. marcescens* T-2000 accumulated 100 g/L of L-threonine using ammonium as a nitrogen source. This value is much higher than those reported previously.

T-2000 harbors the recombinant plasmid of the threonine operon and has the high potential of synthesis from L-aspartate to L-threonine. T-2000 carries five to six copies of the recombinant plasmid and produces threonine synthetic enzymes at a three to four times higher level than host strain T-1165. Nevertheless, T-2000 did not accumulate the expected amount of L-threonine based on the gene dosage. We supposed that this low L-threonine productivity resulted from the slow supply of ammonium, and the slow supply of ammonium was followed by slow incorporation of L-gluatamine and L-aspartate to the threonine synthetic

Table 2
Productivity of L-Threonine in *S. marcescens* T-1165 and T-2000

Strain	Nitrogen source	Culture time, d	Thr produced, g/L
T-1165[a]	Urea	3	40
	Ammonia	2	40
T-2000[b]	Urea	3	55
	Ammonia	2	65

[a]T-1165 = host strain.
[b]T-2000 = recombinant strain.

pathway. Therefore, we attempted to apply the method of ammonium supply to the L-threonine production with recombinant strain T-2000. In the cultivation using urea, L-threonine production with T-2000 increased by only 15 g/L as compared with host strain T-1165 (Table 2). However, when using ammonium as a nitrogen source, L-threonine production increased by 25 g/L in the cultivation. This result showed that this recombinant producer was not able to express its high potential of L-threonine productivity in the cultivation on the urea medium. Some recombinant strains do not express their expected potential based on the gene dosage. One reason for this fact is the shortage of intermediate metabolites as precursors. Improvements of cultivation methods, especially methods of precursor supply, are important for efficient fermentative production with recombinant strains.

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