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Characteristics of L-Phenylalanine and L-Tyrosine Fermentation in Regulatory Mutants of *Corynebacterium glutamicum*

Dong-Il Kim
MOGAM Biotechnology Research Institute, 341 Bojung-Ri, Goosung-Myun, Yongin-Goon, Kyonggi-Do, 449-910, Korea

조절기작을 상실한 *Corynebacterium glutamicum* 변이주의 L-Phenylalanine 및 L-Tyrosine 발효특성

김 동 일
(재) 목암생명공학연구소

ABSTRACT

An auxotrophic regulatory mutant of *Corynebacterium glutamicum* ATCC 21674 produced 2.1 -3.4 g/l of phenylalanine with 2.9-4.4 g/l of tyrosine in the batch shake flask fermentations. At higher sugar concentration, the production of both amino acids was lower than that at low sugar concentration. There was a pronounced effect of temperature on the amino acid production. At 30℃, much higher levels of phenylalanine and tyrosine were produced than those at 37℃. The pH decrease in the shake flask fermentation was so fast that it was impossible to maintain a constant pH with calcium carbonate as a buffering agent. Even though the strains we have used are reported as tyrosine auxotrophs, they produced tyrosine and were able to grow on the minimal medium where no tyrosine was present.

INTRODUCTION

Aspartame is a diamide formed from commonly occurring amino acids, L-aspartic acid and the methyl ester of L-phenylalanine(1). The chemical structure of aspartame is shown in Fig. 1. Although aspartame is derived from amino acids, it in no way reflects the taste of L-aspartic acid or that of L-phenylalanine(2). The former is tasteless and the latter is bitter. To become competitive with other sweetners, the price of the artificial sweetner must be reduced. Manufacturing cost of aspartame depends on several factors. Among them is the price of L-phenylalanine and L-aspartic acid, the two constituent amino acids. If L-phenylalanine can be produced more cheaply, the cost of aspartame can be reduced because L-phenylalanine is much more expensive than L-aspartic acid. Most of the present companies producing L-phenylalanine use a fermentation process that gives a highly impure product. If we can increase the final concentration of phenylalanine in the broth, it will reduce the cost of the amino acid and subsequently of aspartame.

Direct production of L-phenylalanine using tyrosine auxotroph has been investigated since the excretion of L-phenylalanine was first discovered in a tyrosine auxotroph of *Escherichia coli*(3). Adelberg(' discovered that mutants of *Escherichia coli* resistant to thienylalanine, an analogue of phenylalanine, excreted a large amount of phenylalanine, and in some cases smaller amounts of tyr

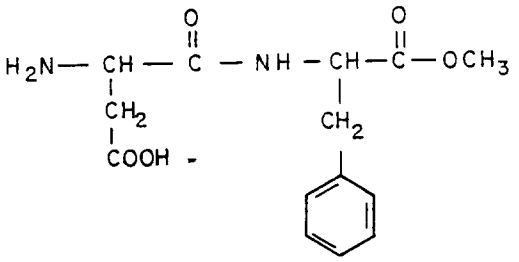

Fig. 1. Chemical structure of aspartame, L-aspartyl-L-phenylalanine methyl ester.

osine. Oishi(5) reported the production of L-phenylalanine using a Cyrosine auxotroph of *Corynebacterium glutamicum* commonly employed for glutamic acid production. The combination of the above two markers, auxotrophy and analogue resistance, was expected to improve the production of L-phenylalanine. In addition, genetic manipulation also increased the level of L-phenylalanine production(6,7).

In this study, batch shake flask cultures of *Corynebacterium glutamicum* producing L-phenylalanine were evaluated. Time courses of cell growth, amino acid production, and sugar consumption were observed at different levels of initial sugar concentrations. The effect of culture temperature was also examined and the comparison of the amino acid production characteristics of four different strains was made.

MATERIALS AND METHODS

Microorganism

Four different strains of *Corynebacterium glutamicum* ATCC 21672, ATCC 21673, ATCC 21674, and ATCC 21677 were purchased from American Type Culture Collection. These strains were isolated as high phenylalanine producer by Nakayama et al. at Kyowa Hakko Kogyo Co., Ltd. in Tokyo, Japan and were deposited in a patent application (8). All these strains are tyrosine auxotrophs, and are resistant to several phenylalanine and tyrosine analogues as shown in Table 1. The exact property of the strain ATCC 21677 is not available. Cultures were maintained for a short period on seed agar slants and transferred monthly.

Culture media

The seed and fermentation media were prepared according to Nakayama et al.(8). The seed medium consisted of 20 g / *l* of glucose, 2.5 g / *l* of NaCl, 10 g / *l* of peptone, and 10 g / *l* of yeast extract. The glucose was autoclaved separately in 10% of the total volume, then aseptically combined. The final pH was 7.2. Agar slants for maintenance were made by adding agar at a concentration of 20 g / *l* to this medium and dispensing into screw top tubes before autoclaving. The composition of fermentation medium was described previously(9). Typically, cane molasses was used as the carbon source. $MgSO_4 \cdot 7H_2O$ was autoclaved separately.

Growth measurement

Growth on seed medium can be measured using a Klett Somerson colorimeter (Arthur Thomas Co., Philadelphia, PA) using a red filter. Cell growth in the fermentation medium was also monitored by measuring the turbidity using a Klett Somerson colorimeter after dilution with distilled water. Dry cell weight was measured and used to calibrate the optical density measurement. In order to measure the dry cell weight, 1 N of HCl was used to dissolve the $CaCO_3$ in the fermentation medium. 10ml of the sample was centrifuged and dissolved in 1 N HCl, and this sample was then washed twice with distilled water. The centrifuged solids were dried at 60℃ overnight and weighed. This method showed good reproducibility for

Table 1. Properties of the microorganisms. Abbreviation of markers are as follows: Tyr^-, tyrosine requiring: PFP^r, resistant to 4-fluorophenyl alanine: TA^r, resistant to β-2-thienylalanine: PAP^r, resistant to 4-aminophenylalanine: $3AT^r$, resistant to 3-aminotyrosine

Strain	ATCC Number	Markers
Corynebacterium glutamicum	21672	Tyr^-, PFP^r, $3AT^r$, TA^r
Corynebacterium glutamicum	21673	Tyr^-, PFP^r, PAP^r, $3AT^r$
Corynebacterium glutamicum	21674	Tyr^-, PFP^r, PAP^r, $3AT^r$, TA^r

cell concentration of 1 g / l and higher.

Sugar analysis

Sugar concentrations in the fermentation broths were measured using a high performance liquid chromatography system (Waters Associates, Bedford, MA) with HP 85X column (Biorad, Richmond, CA). Standards and fermentation samples were prepared in the following manner. 10 ml of the sample was centrifuged and the supernatant was duluted with distilled water. The sample was then filtered through 45 μm Millipore membrane. 2 ml of the prepared sample was dispensed into HPLC vial. 60 μl of the sample was automatically injected into the column at a flow rate of 0.5 ml / min using 5 mM H_2SO_4 as the solvent. By using this HPLC, lactic acid and acetic acid also can be measured.

Amino acid measurement

Phenylalanine and tyrosine concentrations in the fermentation broth were measured using HPLC with μ-bondapack C18 column (Waters Associate part# 27324, 30× 3.9 mm i.d.). The method described by Hill et al.(10) was used.

RESULTS AND DISCUSSION

Batch fermentation with 15% molasses

A batch fermentation was performed in 500 ml shake flasks with 50 ml of medium using *Corynebacterium glutamicum* ATCC 21674. This fermentation lasted four days. The temperature was controlled at 30℃ and the growth of cells and the production of phenylalanine were determined. In this experiment, 15% of molasses which is equivalent to 75 g / l of total reducing sugar was added initially as the carbon source. The results of this experiment are shown in Fig. 2. During the course of the fermentation, 67 g / l of total reducing sugar was consumed. Final cell concentration was about 5,000 Klett units as measured optically. Approximately, 3.4 g / l of phenylalanine and 4.3 g / l of tyrosine were produced after three days of the fermentation and no further increase in the concentration of the amino acid or the cell density was observed.

Batch Fermentation with 23% of molasses

In order to evaluate the effect of total reducing sugar concentration on the cell growth and the formation of the product, another experiment was performed in 500-ml shake flasks. In this fermentation, molasses concentration was increased to 23% which is equivalent to 115 g / l of total reducing suger. The temperature was also main

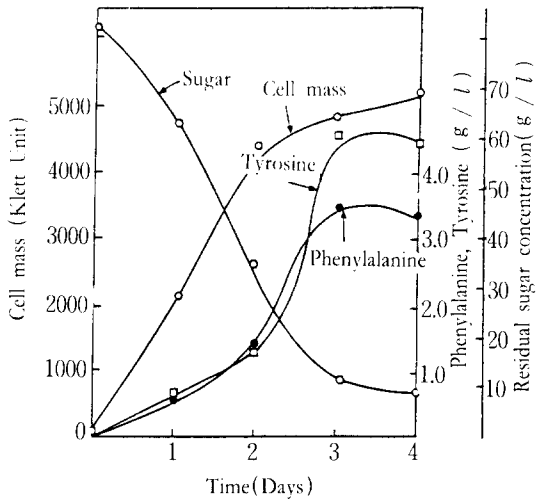

Fig. 2. Time course profiles of L-phenylalanine fermentation in 500-ml shake flasks with 15% molasses medium.

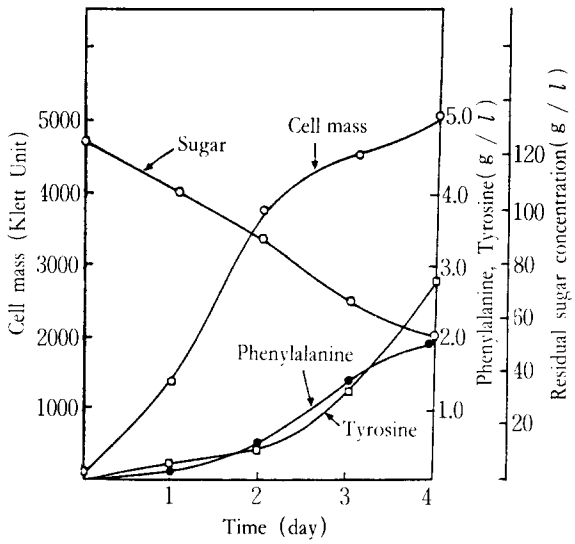

Fig. 3. Time course profiles of L-phenylalanine fermentation in 500-ml shake flasks with 23% molasses midium.

tained at 30℃. The results from the four days of cultivation are shown in Fig. 3. Approximately 2.1 g / l of phenylalanine and 2.9 g / l of tyrosine were produced and 66 g / l of total reducing sugar was consumed at the end of the fermentation. This sugar consumption was almost the same as that of the previous experiment. Final cell concentration was slightly lower than that in the former fermentation. As seen before, more tyrosine was produced than phenylalanine at the end of the fermentation. The concentration of both amino acids, however, increased continuously until the end of the fermentation. From this fermentation, we can infer that high initial sugar concentration was not helpful in improving the production of phenylalanine.

When sucrose was used as the carbon source instead of molasses, the cells grew very slowly and higher concentration of tyrosine was produced than that for phenylalanine. All these results are summarized in Table 2.

Batch fermentation with lower concentration of carbon source

Additional fermentations were also performed in shake flasks to examine the effects of lowering molasses concentration and temperature on the production of phenylalanine by *Corynebacterium glutamicum* ATCC 21674. In these experiments, 10% of molasses was used and temperatures of 30℃ and 37℃ were evaluated. A summary of these results are shown in Table 3. It can be seen that there was a pronounced effect of temperature on the production of the amino acids. At 30℃, 9.91 g / l of tyrosine was produced, accompanied by only 1.13 g / l of phenylalanine. Final cell concentration was about 2,020 Klett units as measured optically. At 37℃, tyrosine concentration was 6.65 g / l and phenylalanine concentration was 0.52 g / l. Final cell concentration was lower than that found at 30℃. It can be seen that the cell growth at both temperatures, 30℃ and 37℃, was significantly lower than those found with higher molasses concentration. In spite of lower cell concentration, the production of tyrosine increased significantly than previously found. It should be noted that the results shown in Table 3 were quite strange in view of the fact that the strain *Corynebacterium glutamicum* ATCC 21674 is supposed to be a tyrosine auxotroph. At 30℃, both cell growth and the production of the amino acids were higher than those at 37℃. It should be noted that in both fermentations, at 30℃ and 37℃, the pH dropped drastically during the course of the fermentation. At both temperatures, the initial pH was 7.2 and the final pH was 4.5 at 30℃ and 4.9 at 37℃. As expected, it was difficult to maintain the pH in shake

Table 2. Effect of carbon sources on the production of L-phenylalanine

Carbon source	Phenylalanine Concentration (g / l)	Tyrosine Concentration (g / l)	Growth (Klett Units)	pH Change	Total sugar consumption (g / l)
15% molasses	3.41	4.38	5,360	rapid	67
23% molasses	2.05	2.88	4,960	rapid	66
5% sucrose	0.30	3.40	1,150	slow	27

Table 3. Temperature effect on the production of L-phenylalanine in shake flasks with 10% molasses medium

Temperature	Phenylalanine Concentration (g / l)	Tyrosine Concentration (g / l)	Growth (Klett Units)	pH Change
30℃	1.13	9.91	2,020	7.2to4.5
37℃	0.52	6.65	1,710	7.2to4.9

flask fermentation.

Even though the calcium carbonate had been used as a buffering reagent, it did not contain sufficient buffering capacity to maintain the pH. During the fermentation, it is also difficult to supply enough oxygen. When the skake flask is used, this oxygen limitation could result. Due to the oxygen deficiency, byproducts such as lactic acid and acetic acid can be produced. In this experiment, more than 4 g / l of lactic acid and 2 g / l of acetic acid were detected at the end of the fermentation.

Production of phenylalanine by four new and different strains of *Corynebacterium glutamicum*

As discussed above, it was found that *Corynebacterium glutamicum* ATCC 21674 produced tyrosine. To examine the auxotrophic property of the strain, minimal medium, where no tyrosine was present, was used. In this experiment, the strain grew on minimal medium leading us to conclude that *Corynebacterium glutamicum* ATCC 21674 used in the previous experiment is not a tyrosine auxotroph which is contrary from the result as reported in the literature(8). Even though the strain is not a tyrosine auxotroph, it was found that it was still resistant to the aromatic amino acid analogues such as p-fluorophenylalanine. Sugimoto et al.(11) reported that a mutant strain of *Brevibacterium flavum* resistant to the phenylalanine analogues accumulated both tyrosine and phenylalanine. Therefore, it appears that the strain lost its auxotrophic property, but still has analogue resistance so that it could produce both phenylalanine and tyrosine at a higher level than the wild type strain. From the above reason, new and different strains of *Corynebacterium glutamicum* were examined to compare the production of tyrosine and phenylalanine in shake flasks. In these experiments, the incubation temperature was 30℃ and the initial molasses concentration was 20%. From the results shown in Table 4, it can be seen that all four strains produced phenylalanine and tyrosine. Except for the ATCC 21672, the levels of tyrosine were slightly higher than that for phenylalanine. Even though all four strains are reported to have the tyrosine auxotrophic properties and analogue resistance, it appears that all these strains have lost their auxotrophic properties.

요 약

본 연구에서는 L-phenylalanine을 생산하는 조절기작을 상실한 영양요구성 변이주인 *Corynebacterium glutamicum* ATCC 21674를 이용하여 플라스크내에서의 회분식 배양시의 특성을 조사하였다. 이 균주는 회분발효시 2.1-3.4 g / l 의 phenylalanine과 2.9-4.4 g / l 의 tyrosine을 생산하였고, 당농도가 높을 경우 생산성이 저하됨을 알 수 있었다. 또한 온도의 변화는 이들 아미노산 생산에 큰 영향을 미침이 관찰되었다. 30℃에서 배양하는 경우, 37℃에서 배양하는 것보다 훨씬 많은 아미노산이 생산되었다. 배양도중 pH는 급격한 변화를 보였다. 이 균주는 tyrosine이 없는 최소배지에서도 자라는 것이 확인되었고, tyrosine을 과량 생성까지 함으로 보아 영양요구 성질을 상실한 revertant로서 조절기작 상실성은 유지한 것으로 판단된다.

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Table 4. L-phenylalanine production with four different new strains

Strain	Phenylalanine Concentration(g / l)	Tyrosine Concentration(g / l)
ATCC21672	2.59	2.18
ATCC21673	2.49	3.10
ATCC21674	2.13	2.76
ATCC21677	1.03	1.76

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