한국생물공학회지 제5권 제2호
*Korean J. Biotechnol. Bioeng.*
Vol. 5. No. 2. 125–131(1990)

# Kinetics of L-Phenylalanine Production by *Corynebacterium glutamicum*

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

# *Corynebacterium glutamicum*에 의한 L-Phenylalanine 생산의 동역학적 특성

김 동 일
(재) 목암생명공학연구소

## ABSTRACT

Microbial production of L-phenylalanine using *Corynebacterium glutamicum* ATCC 21674, a tyrosine auxotroph resistant to aromatic amino acid analogues, has been studied and kinetic analysis was performed. Even though the strain was reported as a tyrosine auxotroph, it produced tyrosine and was able to grow on the minimal medium where no tyrosine was present. The average specific growth rate at the exponential growth phase was 0.087 $hr^{-1}$. There was a dissociation of growth from the formation of the product. Linear correlation between biomass production and total $CO_2$ production was obtained. The relationship between $CO_2$ evolution rate and sugar consumption rate was also found to be linear.

## INTRODUCTION

L-Phenylalanine is an industrially important amino acid. It has been used for medical purposes for quite some time. Not only it is essential for human nutrition, but its methyl ester when combined with L-aspartic acid comprises the non-nutritive sweetner, aspartame. Aspartame was approved by the U.S. Food and Drug Administration as a sugar substitute for table use and as a food flavoring agent in 1981, and it was cleared for use in diet soft drinks in the summer of 1983(1). Recently it was approved in many countries as a food additive and is receiving much attention as a low-calorie sweetner. It is approximately 200 times as sweet as sucrose and has a pleasant sweetness without a bitter after taste(2). Due to the increased use of aspartame as a food additive, the worldwide phenylalanine market increased and the climbing sale has been continuing (3). 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. As the demand for L-phenylalanine has increased, new proceses for its production have been developed using chemical, enzymatic, and fermentative production methods. Several investigators summarized the earlier and recent studies on the production of phenylalanine(4–7).

Until now most of the commercial production of phenylalanine has been attained through microbial fermentations in spite of the difficulties in the purification processes. The aromatic amino acid biosynthetic pathway and its regulation in *Corynebacterium glutamicum* are well known and this strain is widely used for the production of phenylalanine. Hagino and Nakayama(8–9) obtained tyrosine auxotroph

which produced large amount of L-phenylalanine and studied the regulatory properties of the enzymes included in the amino acid biosynthesis in the mutants of *Corynebacterium glutamicum*. The direct microbial fermentation employs auxotrophic and regulatory mutants which are resistant to several aromatic amino acid analogues. 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.

In this study, kinetics of phenylalanine production by *Corynebacterium glutamicum* in a 3 l fermentor was investigated. Exit $CO_2$ concentration was measured by a mass spectrometer, and the correlation between the biomass and $CO_2$ production as well as the correlation between $CO_2$ evolution rate and sugar consumption rate were studied.

## MATERIALS AND METHODS

### Microorganism

*Corynebacterium glutamicum* ATCC 21674 was purchased from American Type Culture Collection. The strain was isolated as high phenylalanine producer by Nakayama *et al.* at Kyowa Hakko Kogyo Co., Ltd. in Tokyo, Japan and was deposited in a patent application(10). The strain was a tyrosine auxotroph and was resistant to several phenylalanine and tyrosine analogues suc as 4-fluorophenylalanine, 4-aminophenylalanine, 3-aminotyrosine, and β-2-thienylalanine($Tyr^-$, $PFP^r$, $PAR^r$, $3AT^r$, $TA^r$). The culture was maintained for a short period on seed agar slants and transferred monthly.

### Culture media

The seed and fermentation medium were prepared according to Nakayama *et al.*(10). 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 is shown in Table 1. Typically, cane molasses was used as the carbon source. $MgSO_4 \cdot 7H_2O$ was autoclaved separately. All other ingredients were sterilized prior to the fermentation at 121℃ for 15 min.

**Table 1. Composition of the fermentation medium for *Corynebacterium glutamicum*.**

| Component | Concentration(g/l) |
|---|---|
| Cane molasses | 200 |
| $K_2HPO_4$ | 0.5 |
| $KH_2PO_4$ | 0.5 |
| $MgSO_4 \cdot 7H_2O$ | 0.25 |
| $(NH_4)_2SO_4$ | 20 |
| Corn steep liquor | 5 |
| $CaCO_3$ | 20 |

Dissolved in distilled water to a final volume of 1 l and the pH was adjusted to 7.2 before autoclaving.

### Culture apparatus

A home-made 3 l fermentor with a working volume of 2 l was used in this work. The pH was controlled by a home-made pH controller with an Ingold pH electrode (Lexington, MA), which was autoclaved inside the fermentor. A variable speed peristaltic pump was used to add base and acid to control the pH. The temperature was connected to by a temperature controller and a tape heater at 30℃. The exit gas line was connected a mass spectrometer to measure to gas concentrations.

### 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. 10 ml

of the sample was centrifuged and dissolved in 1 NHCl, 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 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. Ten ml of the sample was centrifuged and the supernatant was diluted with distilled water. The sample was then filtered through 45$\mu$m Millipore membrane. Two ml of the prepared sample was dispensed into HPLC vial. Sixty $\mu$l of the sample was automatically injected into the column at a flow rate of 0.5ml / 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 $\mu$-bondapack C18 column(Waters Associate part# 27324, 30× 3.9 mm i.d). The method described by Hill *et al.*(11) was used.

### Gas analysis

During the fermentation, $CO_2$and $O_2$ concentrations in the exit gas were determined by using a Perkin-Elmer process mass spectrometer(multiple gas analyzer, MGA 1200). The concentrations of $O_2$and $CO_2$ were recorded at one-half-hour intervals using a software written on a DEC PDP 11 / 23 computer(Digital Equipment Corporation, Maynard, MA).

## RESULTS AND DISCUSSION

### Time course behavior in batch fermentation

A batch fermentation using *Corynebacterium glutamicum* ATCC 21674 was performed to examine the general behavior in a controlled environment. Cane molasses was used as a carbon source. The pH was controlled at 7.2 throughout the fermentation using concentrated ammonium hydroxide and the temperature was maintained at 30℃. The results obtained in this experiment are shown in Fig. 1. The initial reducing sugar concentration was 95 g / l and the sugar consumption was accompanied by the growth of the cells. At the end of the fermentation after 96 hours, less than 10 g / l of the reducing sugar remained. Cell growth was exponential after the initial lag phase of about 10 hours and the cells stopped growing after 70 hours. Cell growth on a semi-log scale is shown in Fig. 2. The average specific growth rate was estimated as 0.087 $hr^{-1}$. A final cell concentration of about 17 g / l was obtained. After approximately 30 hours, tyrosine and phenylalanine excretion started. As shown in Fig. 1, tyrosine was produced continuously until the end of the fermentation reaching a maximum concentration of 10.5 g / l. Even though the cells did not grow during the last 30 hours of the fermentation, the production of tyrosine continued. On the other hand, the final concentration of phenylalanine was only 1.5 g / l. Even though this strain of *Corynebacterium glutamicum* was supposed to be a tyrosine auxotroph, it produced large amount of tyrosine during the fermentation. In addition, the cells were able to grow on the minimal medium where no tyrosine was present and they were still resistant to the aromatic amino acid analogues such as pfluorophenylalanine. Therefore, it appears that the strain lost its auxotrophic property, but still had analogue resistance so that it produced both phenylalanine and tyrosine at higher levels than those of the wild type strain.

Kinetic analysis was performed to examine the general behavior in the batch fermentation. The results from Fig. 1 were used to calculate the specific growth rate, the specific product formation rate, and the sugar consumption rate. Carbon dioxide evolution rate was determined from the exit gas data. By using the above parameters, several correlations which might be useful to increase the yield of the amino acid were obtained.

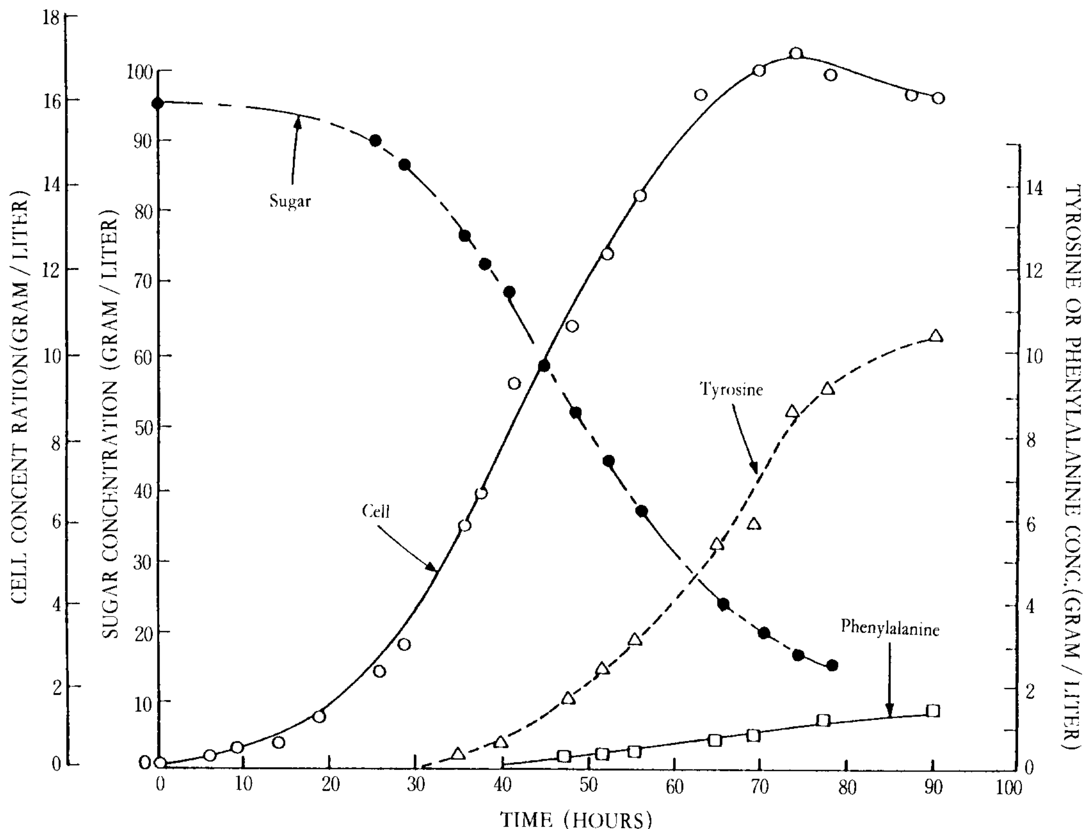


**Fig. 1. Batch fermentation with *Corynebacterium glutamicum* in a 3-liter agitated fermentor.**

## Relationship between specific growth rate and specific product formation rate

Fig. 3 shows the relationship between the specific growth rate and the specific product formation rate. The specific growth rate and the specific product formation rate during the course of the fermentation were normalized to represent fractions of the maximum values. The specific product formation rate was defined as g phenylalaning / g cell-/ hr. Time course behavior of the normalized specific growth rate and the specific product formation rate are shown in Fig. 4. As can be seen from this figure, the maximum specific growth rate was maintained from the 20th to 50th hour of the fermentation. After 50 hours, the specific growth rate decreased continuously up to the 70th hour. On the other hand, the specific product formation rate was maximum between the 70th and 80th hour of the fermentation, but lasted only a short period of time. Even though the cells stopped to grow, the specific product formation rate was high and it decreased continuously until the end of the fermentation. From these results it can be seen that the production of phenylalanine is dissociated from the growth of the microorganism. From the relationship between the specific growth rate and the specific product formation rate which is shown in Fig. 3, it should be noted that the product formation was low at high growth rate. Thisagain shows the evidence of dissociation of the product formation from the cell growth. For a preciser analysis of the effect of growth rate on

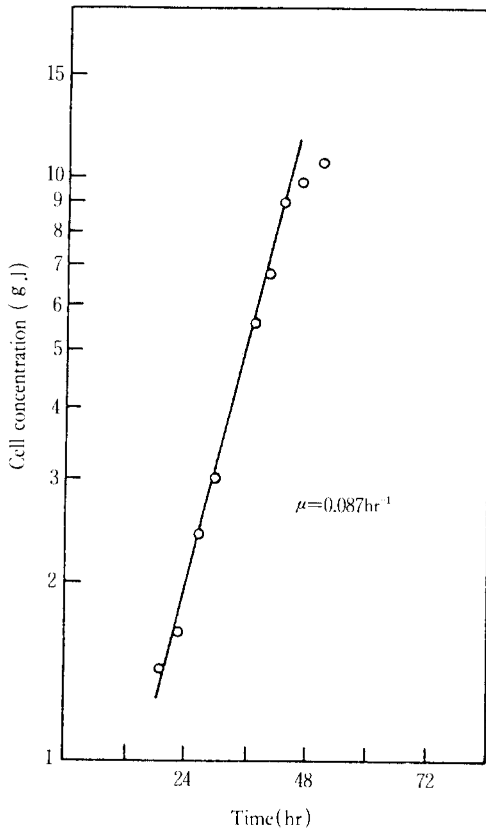


Fig. 2. Cell growth curve on a semi-log scale.

product formation, continuous culture should be performed, but the result shown above seemed to be enough to show the dissociation. In view of the results, it was found that one can control the growth rate, after obtaining high concentration of cells as soon as possible, in order to maximize the formation of the product.

## Correlation between cell growth and carbon dioxide production

The experimental correlation between the cell densty in g/l and the total $CO_2$ production in mmole/l is shown in Fig. 5. Total $CO_2$ production was obtained from the measurement of the exit $CO_2$ concentration. Time

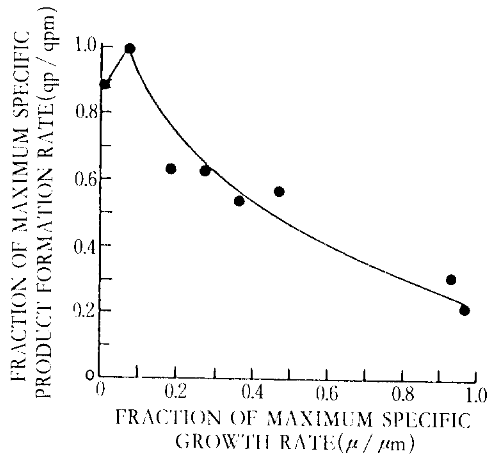


Fig. 3. Relationship between specific growth rate and specific product formation rate.

courses of $CO_2$ and total $CO_2$ production during the course of the fermentation are shown in Fig. 6. As can be seen from Fig. 5, linear correlation was obtained during the growth phase. When the control of the cell concentration or of the growth rate is needed, total $CO_2$ production data which can be obtained from the measurement of the exit gas concentration will be useful to estimate the status of the fermentation.

## Correlation between $CO_2$ evolution rate and the sugar consumption rate

The sugar consumption rate in mmole sugar/l/hr was also calculated from the results shown in Fig. 1. Carbon dioxide evolution rate in mmole $CO_2$/l/hr was obtained from the data shown in Fig. 6. As shown in Fig. 7, the sugar consumption rate was well correlated with the $CO_2$ evolution rate. This linear correlation demonstrated the feasibility of the application of $CO_2$ evolution rate in determining the sugar consumption rate. Therefore, the control of the sugar level during the fermentation can be performed by using the $CO_2$ evolution rate.

## 요 약

본 연구에서는 인공감미료 aspartame의 원료인 L-

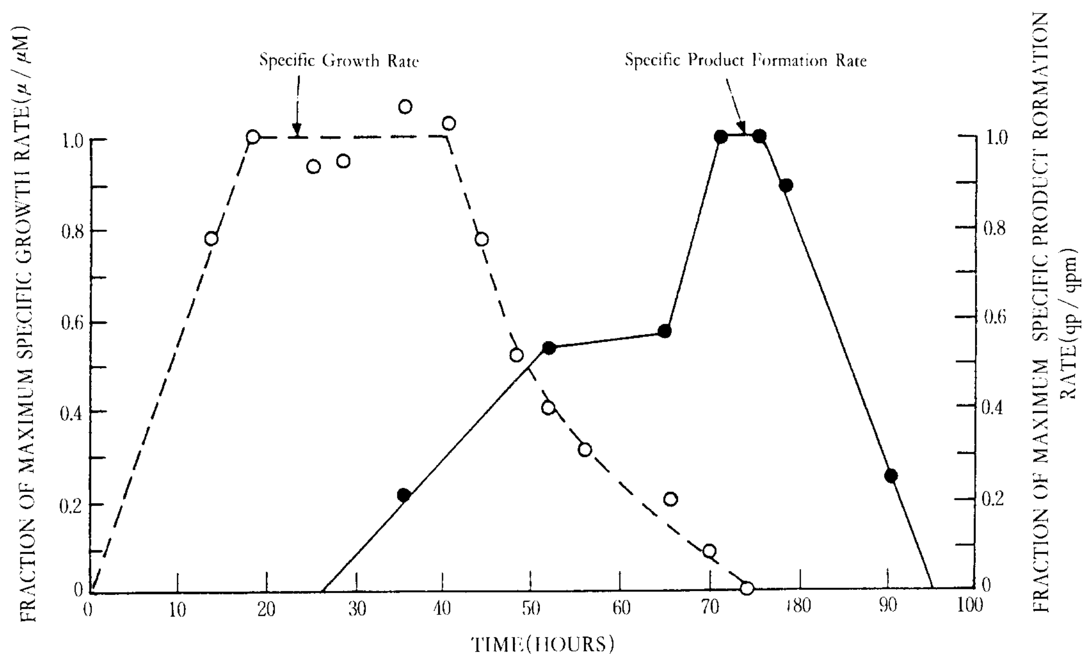


Fig. 4. Kinetics of growth and product formation in agitated fermentor.

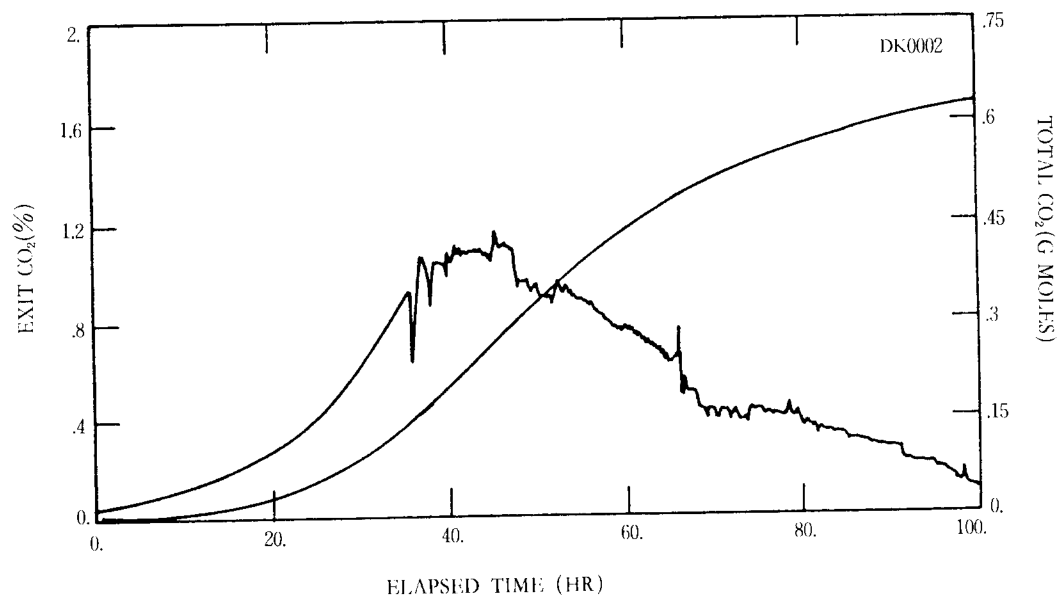


Fig. 6. Time course curve of $CO_2$ concentration during the fermentation.

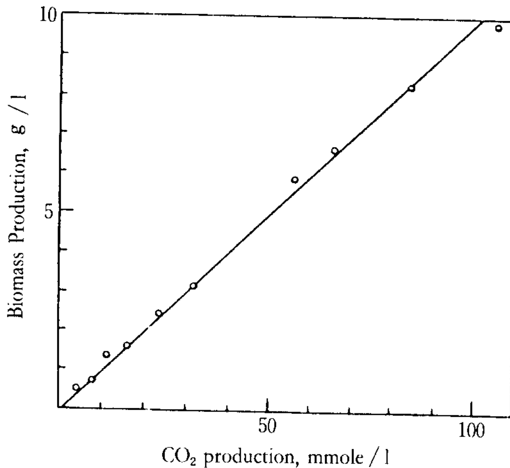


**Fig. 5. Correlation between biomass production and carbon dioxide production.**

phenylalanine을 생산하는 tyrosine auxotroph이며 다수의 아미노산 유도체의 저항성이 있는 변이주 *Corynebacterium glutamicum* ATCC 21674 배양의 동특성을 조사하였다. 이 균주는 tyrosine이 존재하지 않아도 성장하고 또한 과량의 tyrosine을 함께 생성하는 것으로 보아 auxotrophic mutant가 reversion된 revertant로 추정된다. 대수증식기에서의 비증식속도는 0.087hr$^{-1}$이었다. Phenylalanine 최대생성속도는 세포증식이 끝날 때에 얻어졌으며 세포량의 증가는 이산화탄소의 생산량의 증가와 비례함을 알 수 있었다. 이산화탄소 생성속도는 당소비속도와도 비례하므로 이를 이용하여 발효상태를 알 수 있는 유용성이 확인되었다.

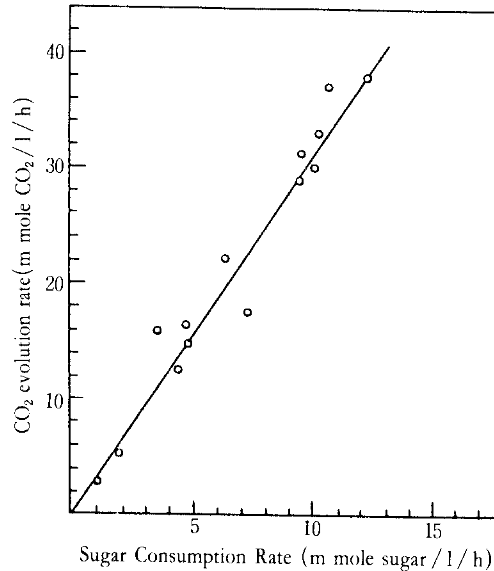


**Fig. 7. Correlation between $CO_2$ evolution rate and sugar consumption rate.**

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**(Received: June 5, 1990, Accepted: July 6, 1990)**