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단보(Note)

Effects of Medium Components on Microbial Production of L-Phenylalanine

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미생물발효에 의한 L-Phenylalanin 생산에 미치는 배지성분의 영향

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ABSTRACT

In this study, effects of medium components on microbial production of L-phenylalanine by *Corynebacterium glutamicum* were investigated. The effect of carbon source on the production of L-phenylalanine was significant. Molasses enhanced the production of L-phenylalanine compared to sucrose, glucose, fructose, or their mixture. It was noticed that trace salts were required for the cell growth and product formation in the minimal medium, but excess amounts of trace salts had no effect on the production of L-phenylalanine. It was also found that optimum amounts of biotin and thiamine were required for the cell growth and the production of L-phenylalanine.

INTRODUCTION

There are several important factors which affect the microbial production of amino acids. The most important step is to obtain high producing strain. Remarkable progress in microbial breeding has made it possible to induce auxotrophic or regulatory mutants that can excrete amino acids. The progress of amino acid fermentations has been remarkable. However, studies mainly dealt with genetic derivation of amino acid product. Even though excellent producers were obtained, the optimization of the culture condition is not easy. Many researchers have studied the influence of the culture conditions to increase the level of the amino acids.

Akashi et al.(1) investigated the influence of oxygen tension on the product formation in phenylalanine, proline, glutamine, and arginine fermentations. They found that the cells excreted the largest amount of phenylalanine when the cell's oxygen demand was inhibited at the "cell's respiration rate / rate of cell's oxygen demand" value of 0.45-0.65. However, lactic acid replaced amino acids as the main product under oxygen deficient conditions.

In the case of substrates, most of the reports were concerned with the production of amino acid from carbohydrates. It was also shown that various microorganisms can utilize hydrocarbons as the sole source of carbon. Tokoro et al.(2) derived a tyrosine auxotroph from a hydrocarbon utilizing bacterium which was found to accumulate a large amount of L-phenylalanine in the broth. Tanaka et al. (3) also demonstrated a process for producing L-phenylalanine by fermentation comprising culturing a hydrocarbon-assimilable and tyrosine requiring microorganism in a medium which contains hydrocarbon as the carbon source.

Suzuki et al.(4) reported the production of aromatic amino acids from methanol by analogue-resistant mutants

of *Methylomonas methanolophila* 6R. In addition to the carbon sources, the effect of L-tyrosine concentration, pH, organic nutrients and precursors on the production of L-phenylalanine was studied by Tokoro et al.(2).

In this study, the effects of carbon sources, trace salts, and vitamins on the production of amino acid were evaluated. In addition, the production of L phenylalanine by microorganisms other than *Corynebacterium glutamicum*, such as *Brevibacterium lactofermenticum* and *Arthrobacter citreus*, was also investigated.

MATERIALS AND METHODS

Microorganism

Corynebactenium glutamicum ATCC21674 was purchased from American Type Culture Collection (Rockville, MD). 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 (5). *Brevibacterium lactofermenticum* ATCC 21420 and *Arthrobacter citreus* ATCC 21422 were also purchased from American Type Culture Collection. These two strains had been deposited by Okumura et al.(6). All these strains are tyrosine auxotrophs, and are resistant to several phenylalanine and tyrosine analogues as shown in Table 1.

Culture media and growth measurement

The seed and fermentation medium for *C. glutamicum* ATCC 21674, and the method for the growth measurement were described previously(7). The culture medium for *B. lactofermenticum* and *A. citreus* were those recommended by Okumura et al.(6) and are shown in Table 2.

Sugar analysis

Dinitrosalycilic acid reagent method (DNS method) was used for the measurement of the total reducing sugar. The sample was first centrifuged and the supernatant was diluted with distilled water to contain 5-14 g / L of total reducing sugar. To 250μL of this sample, 50μL of concentrated HCl was added to hydrolyze the nonreducing sugar. After boiling for 10 min in a water bath, 3mL of DNS reagent was added to 25μL of this sample The absorbance was measured at 575nm using Gilford Model 240 spectrophotometer. Glucose was used as standard.

Table 2. Composition of culture medium for *Brevibacterium lactofermenticum* and *Arthrobacter citreus*

Component	Concentration
Glucose	100 g / L
$(NH_4)_2SO_4$	30 g / L
KH_2PO_4	1.5 g / L
$MgSO_4$	8 g / L
Biotin	10 μg / L
Thiamine HCl	10 μg / L
$MnCl_2 \cdot 4H_2O$	10 mg / L
$FeCl_3 \cdot 6H_2O$	10 mg / L
Corn Steep Liquor	5 mg / L
$CaCO_3$	10 g / L

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.). Anhydrous disodium hydrogen phosphate, 3.549 g, was dissolved in 400mL of double distilled water and the pH was adjusted to 7.2. The resulting solution was diluted to a total volume of 2 L to yield a 0.0125 M Na_2HPO_4 solution, and then filtered through a 0.45 μm Millipore membrane. Saturated borate buffer was made

Table 1. Properties of the microorganisms

Strain	ATCC Number	Markers
Corynebacterium glutamicum	21674	Tyr^-, PFP^r, PAP^r, $3AT^r$, TA^r
Brevibacterium lactofermenticum	21420	Tyr^-, PFP^r
Arthrobacter citreus	21422	Tyr^-, PFP^r

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.

by adding enough boric acid with heat to 1 L of double distilled water. After cooling, the supernatant was filtered and the pH was adjusted to 9.5 with sodium hydroxide. Phenylalanine and tyrosine standards were made at the level of 1 g / L. For the preparation of o-phthaldehyde / ethanethiol derivatizing solution, 50mg of o-phthaldehyde was dissolved in 4.5mL of HPLC grade methanol. Fifty μL of ethanethiol and 9.5 mL of the borate buffer were then added and the solution was mixed. All of these solutions were mixed into a light protected bottle with 20mL of methanol, 10mL of double distilled water, and 9.5mL of borate buffer. The resulting solution was used for only one day. To a 5-mL test tube, 25μL of sample or standards and 2.5mL of o-phthaldehyde / ethanethiol derivatizing solution were added. The mixture was allowed to react at room temperature for 1 min, and then injected by using a Hamilton syringe.

RESULTS AND DISCUSSION

Effects of Carbon Sources on the Production of L-Phenylalanine

In order to examine the effect of each sugar on the production of L-phenylalanine, a series of shake flask fermentation were performed using *Corynebacterium glutamicum* ATCC 21674. These results are shown in Table 3. Total concentration at 100 g / L of each sugar, molasses, or mixture was used. In all cases, between 4 g / L and 6 g / L of tyrosine was obtained. When glucose, fructose, or their mixture was used as a carbon source, less than 1.5 g / L of phenylalanine was produced. On the other hand, 3.26 g / L of phenylalanine was obtained when molasses was used. Molasses contains sucrose, glucose, fructose, and many other unknown nutrients. In view of this result, it appears that the molasses contains unknown ingredients other than sugars, which enhance the production of phenylalanine selectively. In order to examine the reason why molasses enhance the excretion of phenylalanine, the effects of inorganic salts and vitamins were studied.

Effects of Trace Salts on the Production of Amino Acids

Several inorganic ions such as Fe^{2+} and Mn^{2+} are important for high yield of amino acids, in genral. In order to obtain the optimal concentration of trace salts, different concentrations of trace salts were examined. Minimal medium with sucrose as a carbon source was used in this experiment. The experiment was performed using 50mL medium in 500 mL shake flasks for four days. The concentration denoted as 1x was the trace salts concentration recommended by Hagino and Nakayama(8). The results are presented in Table 4. In this series of experiments, it is worthy of notice that the trace salts influenced the production of amino acids. Without the trace salts, there was significant decrease in the production of tyrosine. However, after addition of the inorganic salts, final concentration of tyrosine did not change significantly. Excess amounts of the trace salts had no effect on the production of tyrosine and there was no increase in the concentration of phenylalanine.

Effect of Vitamins on the Production of Amino Acids

According to Kinoshita and Tanaka(9), biotin is the most important medium growth factor for the glutamic acid fermentation. In the glutamic acid fermentation, the concentration of biotin must be less than optimal concentration necessary for the cell growth. Some strains require

Table 3. Effect of various carbon sources on the production of L-phenylalanine by *C. glutamicum* ATCC 21674. The mixture contains sucrose, glucose, and fructose

Carbon Source	Phenylalanine Concentration(g / L)	Tyrosine Concentration(g / L)
Molasses	3.26	4.48
Sucrose	1.50	5.83
Glucose	0.94	5.19
Fructose	0.99	4.22
Mixture	0.67	4.59

thiamine in adition to biotin. It has been reported that the production of glutamic acid as well as the bacterial growth decrease when the amount of thiamine is limited. Because the strain used in this experiment, *Corynebacterium glutamicum* ATCC 21674, was obtained by serial mutation and selection of glutamic acid producing strain, we thought that the concentrations of biotin and thiamine might have some effect on the production of aromatic amino acids. In order to examine this effect, shake flask fermentations were performed with different concentrations of biotin and thiamine. In these experiments, minimal medium with sucrose as a carbon source was used. The concentrations denoted as 1x was 30㎍/L biotin and 10 0mg/L thiamine which was recommended by Hagino and Nakayama(8). Fermentation time was 4 days. As shown in Table 5, the concentrations of biotin and thiamine were found to have an influence on the production of tyrosine. Similarly to the previous findings, the level of phenylalanine was very low. As can be seen in the Table 5, there was a decrease in the production of tyrosine in the absence of biotin and thiamine. Final concentration of tyrosine increased by increasing the concentration of biotin and thiamine three times higher than those recommended by Hagino and Nakayama (8). However, in the presence of excess biotin and thiamine the production of aromatic amino acids is different from the behavior noted for glutamic acid production where the presence of excess biotin inhibits the production of glutamic acid. From the results described above, it was concluded that 90㎍/L of biotin and 30mg/L of thiamine was optimal for the production of tyrosine.

Production of L-Phenylalanine by Microorganisms Other than *Corynebacterium glutamicum*

In addition to *Corynebacterium glutamicum*, many other microorganisms are known to produce phenylalanine. Most of them are both auxotrophic and regulatory mutants. Among them, *Brevibacterium lactofermenticum* ATCC 21420 and *Arthrobacter citreus* ATCC 21422 were chosen for this study because of their availability. The production of L-phenylalanine was examined in 500-mL flasks. The results after four days of fermentation are shown in Table

Table 4. Effect of trace salts on the production of L-phenylalanine. The concentration denoted as 1x was the trace salts concentration as follows: $Na_2B_4O_7H_2O$, 88㎍; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 37㎍; $MnCl_2 \cdot 4H_2O$, 72㎍; $FeCl_3 \cdot 6H_2O$, 970㎍; $ZnSO_4 \cdot 7H_2O$, 8.8㎍; $CuSO_4 \cdot 5H_2O$, 20㎍ in 1 L of medium

Concentration of Trace Salts	Phenylalanine Concentration(g/L)	Tyrosine Concentration(g/L)
0	0	2.40
1x	0.30	4.51
2x	0.30	4.15
6x	0.30	4.44
10x	0.30	4.71

Table 5. Effect of vitamins on the production of L-phenylalanine. The concentration denoted as 1x was 30㎍/L of biotin and 10mg/L of thiamine hydrochloride

Concentration of Vitamins	Phenylalanine Concentration(g/L)	Tyrosine Concentration(g/L)
0	0	3.72
1x	0.30	4.43
3x	0.30	5.17
6x	0.20	4.92
10x	0.30	5.08

Table 6. L-phenylalanine production by *B. lactofermenticum* ATCC 21420 and *A. citreus* ATCC 21422

	B. lactofermenticum ATCC 21420	*A. citreus* ATCC 21422
Phenylalanine Concentration(g / L)	1.31	0.60
Tyrosine Concentration(g / L)	2.10	0.88
Total Sugar Consumption(g / L)	80	53.7
Cell Growth (g / L)	15	8

6. In this experiment, glucose was used as a carbon source and the initial pH was 6.0. Temperature was maintained at 30℃. It can be seen that 1.31 g / L of phenylalanine was produced accompanied by 2.1 g / L of tyrosine in the case of *B. lactofermenticum* ATCC 21420. *A. citreus* ATCC 21422 produced only 0.6 g / L of phenylalanine. The final concentrations of phenylalanine by these two strains were a little lower than those described in the reported literature(6). Furthermore, it can be seen from the results that these two strains, which were reported to be tyrosine auxotroph, produced tyrosine. Therefore it appears that these two strains are not tyrosine auxotrophs.

요 약

본 연구에서는 미생물발효에 의한 L-phenylalanine생산에 미치는 배지조성의 영향을 살펴보았다. 탄소원의 선택은 L-phenylalanine생산에 큰 영향을 미침을 알았다. 자당, 포도당, 과당 혹은 그 혼합물을 탄소원으로 사용할때에 비해 당밀을 사용하는 것이 L-phenylalanine의 생산성을 크게 향상시켜 주었다. 최소배지의 사용시 미량의 염류들이 요구됨을 확인하였으나, 과량으로 첨가하면 더이상의 효과는 없었다. 또한 세포의 증식과 아미노산의 생산에 있어 적정량의 biotin과 thiamine이 필요함도 알 수 있었다.

Corynebacterium 균주외에 L-phenylalanine생산주로 알려진 조절기작을 상실한 영양요구성 변이주인 *Brevibacterium lactofermenticum*과 *Arthrobacter citreus*의 생산능을 조사해본 결과, *Corynebacterium*에 비해 생산성이 낮았으며 역시 영양요구 성질을 잃어버린 것으로 확인되었다.

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