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L-Glutamic Acid 生成菌에 關한 硏究(第 2 報)

—*Brevibacterium ammoniagenes*의 榮養條件에 關하여—

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Studies on L-Glutamic Acid-Producing Bacteria(II)

—On the Nutritional Requirement of *Brevibacterium ammoniagenes*—

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ABSTRACT

Searches for the nutritional requirements of three strains of *Brevibacterium ammoniagenes* reported in the previous paper were carried out with an aim of achieving the striking accumulation of L-glutamic acid and the large multiplication of cells.

It was recognized that all three strains required both biotin and thiamine, together with amino acids such as histidine or cysteine, for their good growth and extracellular L-glutamic acid accumulation. The quantity of biotin required for remarkable growth of these microorganisms was quite different from that for the maximum production of L-glutamic acid. This result, however, did not apply in the case of thiamine. It was also confirmed that, of 18 amino acids, histidine and cysteine were the most effective organic nitrogen sources, while the most available inorganic ammonium salt resulting in a large amount of L-glutamic acid-production and considerable cell growth was found to be only urea.

Maximum accumulation of extracellular L-glutamic acid, more than 50%(w/w) of the initial sugar content, could be obtained from fermentation in the medium containing wheat-bran extract (*Brev. ammoniagenes* T-1 and *Brev. ammoniagenes* Y-2) or rice-bran extract (*Brev. ammoniagenes* YR-2), which confirmed us a possibility that these bacteria might be employed for industrial fermentation of L-glutamic acid.

INTRODUCTION

A large amount of consumption of L-glutamic acid as a seasoning material in a world-wide has stimulated many scientists to pursue the studies on various methods of L-glutamic acid production since the early stage of 1950s. Free from utilizing microbial cells, in the first place, productions of L-glutamic acid were usually attained due to the chemical methods including hydrolysis of proteins of soy-bean cake and wheat, chemical decomposition of pyrolidon-carboxylic acid, and pure chemical synthesis of it through carbide and furfural. These methods, however, did not serve the satisfactory results because of some difficulties accompanied that L-glutamic

acid should be separated from DL-racemic glutamic acid once formed and that the sufficient amount of L-glutamic acid could never be obtained from natural protein resources with facility.

A series of attempts at employing the microorganisms in the L-glutamic acid production, thereafter, began to be made gradually. Resulting from these efforts, the enzymatic methods using microbial enzymes such as transaminase, acylase, and D-glutamic acid oxidase, first of all, were introduced to the L-glutamic acid production. Using transamiase of *Bacterium ketoglutarium* and *E. coli* which is known to play a role in transferring amino group of amino acid to α-ketoglutaric acid, Matsuo *et at.* (1955) and Katagiri (1957) accomplished L-glutamic acid production from α-ketoglutaric acid and hydrolysates of proteins or special amino acid successfully. Sagaguchi(1957) and Michi *et al.* (1954), meanwhile, could obtain L-glutamic acid from DL-acylglutamic acid by using acylase of fungi and from DL-glutamic acid by means of D-glutamic acid oxidase of bacteria respectively.

Although α-ketoglutaric acid could be produced in large quantities by direct fermentation from glucose at that time (Lockwood and Stodola, 1946; Asia *et al.*, 1955), all the cares began to be centered around the important question if the inorganic ammonium salt could be substituted for amino acid as nitrogen sources in order to produce lager quantities of L-glutamic acid as well as to lessen the complicated procedures of cultures. Solution of this problem was worked out by Smythe (1956) who could obtain L-glutamic acid effectively from α-ketoglutaric acid and citric acid by using inorganic ammonium salt and L-glutamic acid decarboxylase contained in yeast cells. At present most of L-glutamic acid fermentation are achieved due to action of intracellular L-glutamic acid decarboxylase.

The fermentative production of high yields of α-ketoglutaric acid from hexose led to an efficient microbial conversion to L-glutamate (Otsuka *et al.*, 1957; Katagiri *et al.*, 1957; Pfizer and Company, Inc., 1957). Later a number of instances of direct fermentative productions of L-glutamate from sugars and inorganic nitrogen sources were reported (Asai *et al.*, 1957; Kinoshita *et al.*, 1957; Kita, 1957). Encouraging isolations of new L-glutamic acid-producing bacteria such as *Micrococcus glutamicus* (Kinoshita *et al.*, 1958) and *Micrococcus varians* (Asai *et al.*, 1957) enable us to obtain extraordinary amount of L-glutamic acid direcily from glucose by means of L-glutamic acid decarboxylase of those cells.

And now the current tendency is under direction that instead of sugars organic acid, hydrocarbon and aromatic compound are likely to be used as substrates in L-glutamic acid fermentation.

The accurate mechanism of extracellular L-glutamic acid accumulation in the medium is not yet apparent but now Oishi's explanation (1964) is generally allowed to express the most reasonable demonstration. The mechanism can be, in a word, summarized as following: α-ketoglutaric acid, the intermedial products of TCA cycle, is converted to L-glutamic acid due to accepting amino group from excessive nitrogen source by reductive reaction of L-glutamic acid

decarboxylase. During the process of this reaction, biotin is believed to play a very significant role but its proper role is not proved accurately until now. According to the reports of Tanaka and Kinoshita (1960), biotin is suggested to influence on the catabolic fate of glucose. On the other hand, in the latter report of Oishi (1964), it was demonstrated that accumulation of L-glutamic acid in the medium depended upon the permeability of cell membrane system of which action is influenced by biotin content existing in the medium.

In considerations of these reports, nutritional requirement of three strains for the bacterial growth and L-glutamic acid production were examined under various conditions and the results obtained are to be reported in this paper.

Table 1. Composition of various media used

Chemicals(%)	Slant medium	Preculture medium	Medium I	Medium II
Glucose			2.0	5.0
K_2HPO_4			0.1	0.2
$MgSO_4 \cdot 7H_2O$			0.03	0.02
$FeSO_4 \cdot 7H_2O$			0.001	0.001
Urea			0.8	1.2
Yeast Ext.	0.5			
Beef Ext.	0.5	1.0		
Peptone	1.0	1.0		
NaCl	0.25	0.5		
Agar	2.0			
*p*H		7.0—7.5		

Preculture medium was used for the preparation of inoculum.
Medium I is the basal medium used for only measurement of cell growth.
Medium II is the basal medium used for L-glutamic acid fermentation.

MATERIALS AND METHODS

1. Organisms

Three strains of *Brevibacterium ammoniagenes* reported in previous paper were used in this experiment. Pure cultures of these organisms were maintained on agar slope and stored in a refrigerator at 4°C. They were transferred weekly.

2. Medium used

The compositions of various media including agar slant are shown in Table 1. Urea was sterilized separately by filtration with Seitz filter and then added to the basal medium. Glucose and other mineral salts were sterilized respectively by autoclaving at 121°C for 15 mins in order to prevent the medium from caramelizing, and then they were mixed with the basal medium in the aseptical culture tube. Separate sterilizations of another substances such as amino acids, vitamins, and natural nutrients were also carried out by autoclaving or filtration. The *p*H of all media was always adjusted to 7.0—7.5 with 1N HCl and 1N H_2SO_4. The concentrations of various kinds of vitamins and amino acids used are exhibited in Table 2.

3. Inoculum

Inoculum for all nutritional requirement tests was prepared by cultivation in the preculture medium for 18—24 hrs. One loopful of organisms on agar slant was inoculated in 500m*l* of pre-culture medium in 500m*l* Erlenmeyer flask and incubated on a reciprocal shaker (112 rpm) at 30°C for 18 to 24 hrs. After incubation, the bacterial cell gathered by centrifugation (3,000—4,000rpm) at 4°C with International model PR-2 refrigerated centrifuge. The gathered cells were washed three or four times with 0.9% sterilized saline water. After final centirfugation, the washed cells were dispensed in 10m*l* of 0.9% saline water, and then 0.15m*l* of this cell suspension was always added to 6m*l* of every medium as an inoculum. This inoculum was always stored at 4°C and employed within 24 hrs, after then, the newly prepared inoculum was utilized.

4. Cultures

Cultures were always attained with 200-by 21-mm tubes where the medium was dispensed in 6m*l* quantity.

In order to measure only the cell growth, the organisms were cultured in basal medium I supplemented with required substances on a reciprocal shaker (275 rpm) at 30°C for 24 hrs. For the determination of L-glutamic acid-production, the organisms were cultured in basal medium II under the same condition described above except for the culture time, for 72 hrs.

Measurement of cell growth, assay of L-glutamic acid and determination of residual sugar were checked by the same methods as reported in previous paper (Hong *et al.*, 1974).

Table 2. Concentration of various vitamins and amino acids used

Vitamins	Conc.	Vitamins	Conc.
Thiamine HCl	1mg/*l*	Inositol	5mg/*l*
Biotin	5γ/*l*	Folic acid	0.5mg/*l*
Ca-pantothenic acid	1mg/*l*	Glutathione	1mg/*l*
Riboflavin	0.5mg/*l*	Betaine HCl	1mg/*l*
PABA	2mg/*l*	Amino acids	
Niacin	1mg/*l*	L-type	0.05%
Pyridoxine HCl	0.5mg/*l*	D-type	0.1%

5. Determination of lactic acid

Lactic acid was determined according to the procedure of Barker and Summerson (1941).

RESULTS AND DISCUSSION

1. Vitamin and amino acid requirement

A preliminary search for the nutritionally active factor was performed by the addition of casamino acid and vitamin mixtures to the basal medium I. As revealed in Table 3, the very poor growth of cell population occurred when cells of all three strains were incubated

Table 3. Effects of vitamins and casamino acid on the cell growth

Medium	Strains					
	T—1		YR—2		Y—2	
	O.D.	R	O.D.	R	O.D.	R
B	0.170	100	0.212	100	0.173	100
B+V	0.252	148	0.317	150	0.312	180
B+C	0.223	132	0.239	113	0.297	172
B+VC	1.403	825	1.300	613	1.263	730

B: Basal medium I. B+V: Vitamin mixtures are added to the medium I. B+C: Vitamin-free casamino acid is added to the medium I. B+VC: Vitamin mixtures and casamino acid are added to the medium I.

$$R = \frac{\text{O.D. of cells grown in vitamin or amino acid-added medium}}{\text{O.D. of cells grown in basal medium I}} \times 100$$

in basal medium I. In case that either vitamin or casamino acid was singly added to the basal medium I, likewise, cell propagations did not almost appear. Even in case that individual amino acid was added instead of casamino acid, the same result was obtained.

Only simultaneous addition of both vitamin and amino acid to the basal medium could prompt the rate of cell growth over 6 to 8 times to the growth rate in the basal medium I. This result provided the apparent evidence that all of the strains required not only vitamin but also amino acid as nutritional substances for their good growth, and therefore the specific amino acids and vitamins requirement were determined.

1) Amino acid requirement

In order to detect the most available amino acid for conspicuous increment of cell growth as well as for raising the capability of L-glutamic acid-synthesis, every 18 amino acids was examined respectively in the medium II supplemented with vitamin mixtures and the data obtained are presented in Table 4. Among all amino acids, cysteine and histidine were ascertained to function as the most effective factor on promoting the cell-propagations and L-glutamic acid-accumulation. All three strains, of course, showed some differences between these two amino acids in efficacies accelerating the L-glutamic acid-production and cell growth. In

Table 4. Effects of amino acids on the cell growth and L-glutamic acid in the presence of vitamin mixture*

Amino acids	T−1			YR−2			Y−2		
	*p*H	mg wet cells/m*l*	G.A. mg/m*l*	*p*H	mg wet cells/m*l*	G.A. mg/m*l*	*p*H	mg wet cells/m*l*	G.A. mg/m*l*
Ala	9.1	5.40	4.05	9.1	5.72	4.27	9.1	5.99	4.21
Val	9.0	6.27	4.42	8.9	9.33	4.54	9.1	6.40	4.29
Leu	9.4	4.67	3.75	9.2	5.73	3.94	9.3	5.00	3.51
Ile	9.4	3.53	2.52	9.5	1.53	1.01	9.4	3.27	2.14
Pro	9.4	2.47	2.04	9.5	2.41	2.00	9.4	5.33	3.72
Phe	9.3	4.23	2.85	9.3	4.80	3.52	9.2	4.90	3.47
Trp	9.4	4.23	2.42	9.3	5.23	3.99	9.4	2.71	1.99
Met	9.2	5.40	3.47	9.2	5.85	4.33	9.2	5.99	4.12
Gly	8.9	11.66	6.88	9.0	6.33	5.93	9.4	1.21	3.00
Ser	9.2	5.82	4.27	9.3	5.60	3.87	9.2	5.40	3.46
Thr	9.1	5.00	3.82	9.1	14.66	7.52	9.2	3.60	2.51
Cys	8.9	16.33	9.75	8.7	21.67	16.85	8.2	20.67	18.40
Tyr	9.1	15.85	7.88	9.0	13.96	9.21	9.1	14.22	9.18
Asp	9.0	10.00	6.08	9.2	10.02	6.36	9.2	9.53	6.56
Asn	9.2	4.83	3.05	9.1	5.31	3.14	9.4	2.47	1.91
Lys	9.3	5.40	3.52	9.3	5.60	3.89	9.4	5.30	3.19
Arg	9.2	5.11	4.08	9.2	6.00	4.37	9.3	4.44	3.13
His	9.0	19.33	12.50	9.0	15.93	10.92	8.9	15.33	12.85

* Vitamin mixtures are containing all the vitamins as shown in Table 2. Every amino acid is added to the basal medium II.

Brev. ammoniagenes T-1 was histidine clarified to be by far more effective of the two in respect of increasing the cell growth and L-glutamic acid production, but in cases of other two strains, of histidine and cysteine, the latter was the more effective. Though tyrosine and aspartic acid were also proved to elevate the cell propagation to some extent, their elevating efficacies were lower than in the cases of histidine and cysteine. Cells of *Brev. ammoniagenes* T-1 and *Brev. ammoniagenes* YR-2 could multiply increasingly even in the presence of glycine and threonine but the superior results to figures shown in Table 4 were never attained in any cases tested. It was, however, of interest that our strains could be stimulated to multiply largely and synthesize a great quantity of L-glutamic acid to a same extent, even though whichever amino acid of the two, histidine and cysteine, was added to the medium.

Table 5 shows the effects of the concentration of histidine, cysteine, and tyrosine on the cell growth and L-glutamic acid-synthesis in the synthetic medium containing both biotin (1γ/l) and thiamine (1mg/l). Though each optimal concentration of three amino acids required by all strains appeared to vary with strains, as a whole, histidine and cysteine gave more satisfactory effect to the L-glutamic acid-production than tyrosine relatively. It can be also made clear from Table 5 that histidine causes *Brev. ammoniagenes* T-1 to yield much higher rate of L-glutamic acid-accumulation (24.2mg/ml) than eysteine, but in *Brev. ammoniagenes* YR-2 and *Brev. ammoniagenes* Y-2 only slightly appreciable differences between histidine and cysteine. in effects to elevate L-glutamic acid-production exist As for their optimal content required for maximum L-glutamic acid accumulation, however, these two amino acids showed a remarkable difference each other. On the other word, the optimum concentration of histidine required for maximum synthesis of L-glutamic acid followed by maximum cell growth was different from that of cysteine, that is, the former was 0.1—0.2% and the latter was 0.025—0.05%.

At the concentration of histidine more than 0.2%, none of three strains showed the increments of cell growth and L-glutamic acid accumulation.

These properties of our strains described above were quite similar to that of *Brev.* sp. 470—2 (Kida *et al.*, 1961) in view of requiring histidine and cysteine for their good growth and marked accumulation of L-glutamic acid. *Brev.* sp. 470—2, however, showed a slightly different character that tyrosine and aspartic acid could also stimulate the L-glutamic acid accumulation analogous to histidine and cysteine.

From these all experiences, it could be confirmed that, unlike in the case of vitamin such as biotin, the concentration of amino acid essential for L-glutamic acid formation was agreeable to that for the maximum growth of cell population. In other word, the synthesis rate of L-glutamic acid was proportional to the multiplication rate of cells concerned. Resulting from this fact, it was suggested that a large amount of L-glutamic acid could be accumulated only in proportion to the growth rate of cell popu-

Table 5. Effects of the concentration of histidine, cysteine, and tyrosine on the cell growth and L-glutamic acid production.

Amino acids	Conc. (%)	T—1			YR—2			Y—2		
		pH	mg wet cells/ml	G.A. mg/ml	pH	mg wet cells/ml	G.A. mg/ml	pH	mg wet cells/ml	G.A. mg/ml
Histidine	0.0125	9.2	12.20	4.95	9.2	7.30	3.15	8.8	13.39	8.90
	0.025	9.0	14.20	7.00	9.1	12.00	5.10	9.3	15.27	10.75
	0.05	9.0	19.33	9.35	9.0	15.93	9.25	8.9	14.33	12.85
	0.10	7.0	24.00	24.20	8.6	20.20	11.15	8.3	21.00	18.75
	0.20	8.9	15.67	13.00	8.5	24.67	19.25	8.6	20.87	19.70
Cysteine	0.0125	9.1	15.00	8.20	9.0	12.47	6.50	8.8	12.85	7.00
	0.025	8.4	15.80	17.05	8.8	24.00	19.60	8.5	17.60	16.50
	0.05	9.1	16.33	9.75	8.7	21.61	16.85	8.2	20.67	18.40
	0.10	9.0	15.00	10.00	9.0	20.67	7.50	9.0	16.60	11.30
Tyrosine	0.025	9.2	6.02	5.55	9.3	4.07	4.51	9.[illegible]	5.18	4.51
	0.05	9.1	5.85	5.88	9.0	7.48	9.21	9.1	9.61	7.18
	0.10	8.9	12.66	13.05	9.0	8.58	13.32	8.9	17.03	15.93
	0.20	9.4	3.19	8.49	9.2	5.97	11.10	9.0	4.98	13.58
H+C		5.7	37.07	21.65	5.0	28.20	22.15	5.7	37.07	21.65

Each amino acid was added in medium II supplemented with biotin (1γ/l) and thiamine (1mg/l).
H+C: Both histidine (0.1%) and cysteine (0.025%) were added.

lation which was increased due to the addition of amino acid required. Therefore, it was also assumed that these essential amino acids were possibly concerned in intracellular mechanism stimulating the cell growth rather than L-glutamic acid production. Such a thought was supported by the fact that when mixtures of histidine and cysteine were added to the medium, the marked increment in cell propagations appeared, while merely slight increase in L-glutamic acid accumulation occurred as compared with in the case of one of the two addition. On the basis of the fact that the exclusion of these amino acids from the medium caused the poorly limited growth of cell population in spite of the presence of vitamin, all three organisms tested were considered to lack the capability of synthesizing these amino acid or to possess the extremely limited capability.

Why histidine and cysteine can be substituted for each other in spite of the remarkable difference in their molecular structures and intracellular metabolism within the cell, and what roles these amino acids play in bacteriological metabolism, were not to be ascertained in this paper but are very interesting problems which will be solved in the future to come.

2) Vitamin requirement

Which vitamins would be prerequisite to the cell growth and L-glutamic acid production in three strains was detected preliminarily by using the medium containing casamino acid. As shown in Table 6, it can be recognized that both biotin

Table 6. Effects of vitamins grouped on the cell growth in the presence of casamino acid

Medium	T—1		YR—2		Y—2	
	O.D.	R	O.D.	R	O.D.	R
BA+V	1.406	764.1	1.060	493.0	1.223	675.7
BA+V—1	1.022	555.4	0.964	448.8	1.193	659.1
BA+V—2	0.292	158.7	0.214	99.5	0.290	160.2
BA+V—3	0.272	147.8	0.292	135.8	0.270	149.2
BA+V—4	0.263	142.9	0.237	110.2	0.285	157.5
BA	0.184	100.0	0.215	100.0	0.181	100.0

BA: Basal medium I containing 0.05% casamino acid.
V—1: Biotin and thiamine.
V—2: Ca-pantothenic acid, riboflavin, and niacin.
V—3: PABA and pyridoxine.
V—4: Inositol, glutathione, betaine and folic acid.

$$R=\frac{\text{O.D. of cells grown in vitamin-added medium}}{\text{O.D. of cells grown in vitamin-free medium}}\times 100$$

and thiamine were possibly responsible for L-glutamic acid synthesis and the cell growth, i.e., they were assumed to function as an indispensable component to the bacterial cell growth. Other vitamins including Ca-pantothenic acid, riboflavin, *p*-aminobenzoic acid, and pyridoxine were clarified not to have an remarkable effect on the cell growth. In previous paper, since such a vitamin requirement was already investigated, only their effecting ranges were detected in this paper. As shown in Table 7, there existed a difference between biotin and thiamine in the effects on the L-glutamic acid production and cell growth. In the biotin-deficient medium but containing thiamine, all three strains exhibited extremely poor cell growth together with corresponding serious decrease in the quantity of L-glutamic acid produced by about 30—80%, when compared to the case that biotin was supplied at the optimum concentration. From this result, it was confirmed that biotin surely played a significant role on the cell growth as well as L-glutamic acid production.

When thiamine was eliminated from the medium, however, there occurred decreases both in the cell growth and in L-glutamic acid production but its decreasing rate was not so considerable as in the case of biotin. In this case, at any rate, it could be noted that a sum of L-glutamic acid accumulated in the presence of thiamine was usually proportional to that of cell population multiplicated. Such revealed the antithetical effect to that of biotin.

Three strains, furthermore, showed markedly different sensitivities to the cell growth and L-glutamic acid accumulation. *Brev. ammoniagenes* T-1 exhibited the considerable decrease in the cell growth by about 70% in the thiamine-deficient medium, and the accumulation of L-glutamic acid reached its maximum value at the concentration of 0.1mg/*l*, while on the other hand, other two strains could produce the maximum L-glutamic acid at the concentration of

Table 7. Effect of the concentration of biotin and thiamine on the cell growth and L-glutamic acid production

Vitamins	Conc. of vitamins	mg wet cells/m*l*			G.A. mg/m*l*		
		Brev. T—1	*Brev.* YR—2	*Brev.* Y—2	*Brev.* T—1	*Brev.* YR—2	*Brev.* Y—2
*Biotin	No add.	3.58	3.83	3.07	4.25	4.75	3.10
	0.5γ/*l*	8.20	10.33	9.20	5.90	8.50	7.25
	1.0γ/*l*	16.17	16.09	15.87	9.75	14.04	14.17
	2.5γ/*l*	18.01	19.27	21.67	7.46	10.41	16.34
	5.0γ/*l*	18.87	23.20	24.86	6.05	8.03	9.49
	10 γ/*l*	22.40	55.84	38.87	3.42	4.94	6.46
	20 γ/*l*	12.33	26.00	18.04	4.94	5.41	4.75
**Thiamine	No add.	6.20	11.67	18.33	3.80	2.85	3.89
	0.1mg/*l*	19.67	9.87	18.80	7.61	4.41	6.65
	0.5mg/*l*	18.00	11.66	22.33	7.42	6.47	8.83
	1 mg/*l*	18.84	23.00	24.82	6.03	8.17	9.60
	5 mg/*l*	6.07	19.33	20.40	3.65	6.03	4.60

* Biotin is added to the synthetic medium containing histidine (0.05%) and thiamine (1mg/*l*).

** Thiamine is added to the synthetic medium containing histidine (0.05%) and biotin (5γ/*l*).

1mg/*l*.

Biotin, on the contrary to thiamine, exhibited the noticeable results that the optimal content of biotin for the maximum accumulation of L-glutamic acid was aberrant from that for maximum cell growth. The greatest amount of L-glutamic acid was produced in the cultures of all three strains under the limited concentration of biotin(1—2.5γ/*l*), but the multiplication of microorganisms was decreased markedly in this circumstances. Under the excessive content of biotin (10γ/*l*), while on the other hand, the converse result that the outstanding increment of cell propagation was accompanied with a serious decrease in L-glutamic acid production was obtained. It was also an interesting point of view that, apart from other L-glutamic acid producers (*micrococcus glutamicus*), the cell growth of our strains was rather inhibited under the highly excessive biotin concentration approximately more than 20γ/*l*. It was consequently recognized that the level of biotin is much more critical for maximum synthesis of L-glutamic acid than the level of thiamine and such was regarded apparently as a very noticeable characters of these microorganisms tested. Such a conspicuous difference between the concentration of biotin required for maximum growth of cells of our strains and that for maximum production of L-glutamic acid can be said to be one of the important characters of usual L-glutamic acid-producing bacteria, as can be seen in the reports of Kinoshita *et al.*, (1960) and Oishi *et al* (1964).

Under the excessive content of biotin, together with large multiplication of cell population, lactic acid was accumulated in the medium in large quantities. This increment of lactic acid accompanied by the marked cell growth indica-

ted that most of glucose were used up as an energy source for the principal propagation of bacterial cell without converting to L-glutamic acid, and subsequently lactic acid was accumulated in the culture medium as an end product of cell metabolism. It is a questionable issue whether all glucose were completely oxidized in EMP pathway or HMP pathway without converting to L-glutamic acid or some of glucose first converted to L-glutamic acid and then this L-glutamic acid once formed was used for cell growth.

Physiological efficacy of L-glutamic acid synthesis by our strains was at its maximum in cells of an even lower biotin content and the striking regulation of cell synthesis vs. glutamic acid synthesis mediated by biotin suggested an important role for this vitamin in one or more "switching" reaction in bacteria tested.

As a result, it could be elucidated that all three strains reported in previous paper have the characteristics requiring special vitamins and special amino acids for their good growth and L-glutamic acid accumulation in large quantities.

2. Nitrogen sources as nutrients

Table 9 shows the influences of various kinds of inorganic ammonium salts (can be seen in Table 8) on the cell growth and L-glutamic acid production. From this result, it was found that urea was the most favorable inorganic nitrogen source both for the good cell growth and for L-glutamic acid synthesis in all strains. Such characters of our atrains accorded with the bacteriological property that our strains possessed the extremely high activities of urease.

Although $(NH_4)H_2PO_4$ could act as a more effective factor for the striking increment of cell population than urea, the production rate of L-glutamic acid was seriously negligible. This result indicated that $(NH_4)H_2PO_4$ was not suitable nitrogen source for L-glutamic acid production. This unique character requiring only urea can be said to be an unusual property of bacteria which is rarely observed in other species producing L-glutamic acid. Of all *Brevibacterium* species, *Brev.* sp. No.13 (Hattori *et al.*, 1965) shows very similar character to our strains in view of requiring limited ammonium salt, urea. Even though there are some species incapable of utilizing urea as nitrogen source (*Micrococcus varians*, Asai *et al.*, 1957), most of L-glutamic acid producers have an capacity of using not only urea but also other ammonium salts such as NH_4Cl, $(NH_4)_2SO_4$ (*Micrococcus glutamicus*), and ammonium tartrate (*Brev. divaricatum*).

Effect of the concentration of urea on the cell growth, L-glutamic acid accumulation, residual sugar content, and final *p*H are illustrated in Figure 1, 2,

Table 8. Inorganic nitrogen sources used

Nitrogen compound	mol. wt.	nitrogen%	Conc. of N-source (%)
NH_4Cl	53.50	26.17	2.11
$(NH_4)NO_3$	80.05	24.98	1.58
$(NH_4)_2SO_4$	132.15	21.18	2.61
KNO_3	101.11	13.85	3.79
Urea	60.06	46.02	1.20
$(NH_4)H_2PO_4$	115.04	12.17	4.54
NH_4-Tart.	184.15	15.21	3.63
NH_4-Acet.	77.08	18.16	3.04
NH_4-Cit.	226.19	12.38	4.46

Table 9. Effects of inorganic nitrogen sources on the cell growth and L-glutamic acid production

N-sources	T—1		YR—2		Y—2	
	mg wet cells/ml	G.A. mg/ml	mg wet cells/ml	G.A. mg/ml	mg wet cells/ml	G.A. mg/ml
NH_4Cl	6.00	Trace	5.62	0.00	5.81	0.00
$(NH_4)NO_3$	11.40	〃	10.20	Trace	9.60	Trace
$(NH_4)_2SO_4$	5.92	〃	6.01	〃	6.04	〃
KNO_3	3.13	〃	3.63	〃	3.12	〃
Urea	29.20	15.53	21.46	13.02	28.33	20.47
$(NH_4)H_2PO_4$	33.40	Trace	30.63	Trace	30.33	Trace
NH_4-Tart.	5.20	〃	4.96	〃	4.90	〃
NH_4-Acet.			No growth			
NH_4-Cit.	2.00	0.00	1.80	0.00	2.13	0.00

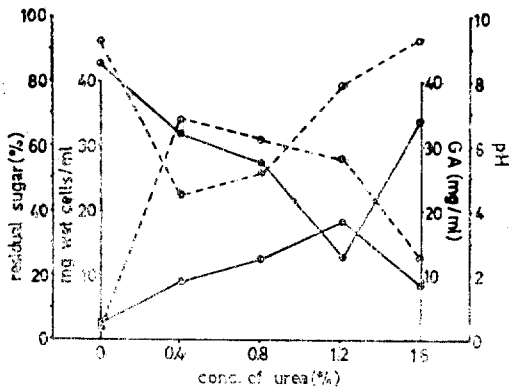

Fig. 1. Effects of the concentration of urea on the cell growth, L-glutamic acid-production, residual sugar, and *p*H in *Brev. ammoniagenes* T—1
Symbols: -○- =L-GA, -•- =residual sugar, - - - =cell growth, --•-- =*p*H.

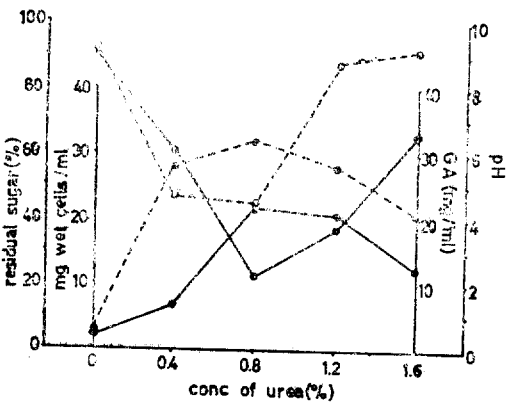

Fig. 2. Effects of the concentration of urea on the cell growth, L-GA production, residual sugar, and *p*H in *Brev. ammoniagenes* YR—2
Symbols: -○- =L-GA, -•- =residual sugar, --○-- =cell growth, and --•-- =*p*H.

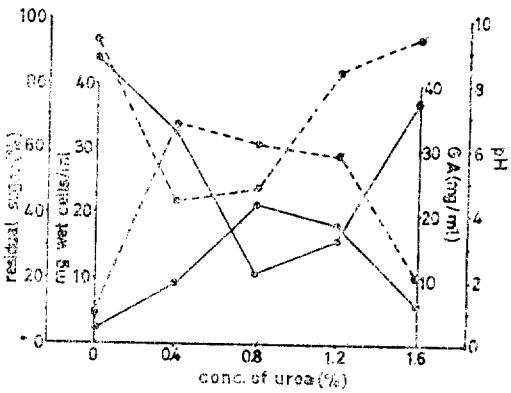

Fig. 3. Effects of the concentration of urea on the cell growth, L-GA production, residual sugar, and *p*H in *Brev. ammoniagenes* Y—2
Symbols: -○- =L-GA, -•- =residual sugar, --○-- =cell growth, and --•-- =*p*H.

and 3. The optimal concentration of urea needed for maximum synthesis of L-glutamic acid has been reported to vary between 0.5% and 1.5% according to the species of L-glutamic acid producers. In cases of bacteria examined in our laboratory, the optimal concentrations appeared to be 0.8% (w/w) in *Brev. ammoniagenes* T-1 and *Brev. ammonia-*

genes YR-2, but in *Brev. ammoniagenes* Y-2 it was 1.2%. This result applied in the case of the growth of cell population. Meanwhile, cell growth of all three strains had reached their maximum values at the concentration of 0.4% urea. It is, of course, taken for granted that there was difference between the time period required for good cell growth and maximum accumulation of L-glutamic acid. At the concentration of 0.4% urea, most of L-glutamic acid once formed from NH_4^+ and glucose by means of the reversible action of L-glutamic acid decarboxylase through transamination reaction, are thought to be utilized for donating amino group for the synthesis of all amino acids reqiured for cell multiplication than to be accumulated in the medium. In order to obtain a large amount of L-glutamic acid effectively, preparation of environmental condition provided with sufficient nitrogen sources is necessarily prerequisite to L-glutamic acid fermentation even in our strains.

As can be already seen in Figure 1,2 and 3, it was also noted that an inverse relation exists between gross accumulation of extracellular L-glutamic acid and gross consumption of sugar supplemented. Such an inverse relation supported the fact that the production of extracellular L-glutamic acid by all three strains is primarily the result of active assimilation of sugar and synthesis of L-glutamic acid but not an autolytic degradation process involving a polypeptide intermediates.

Urea, once added to the medium, is possibly fermented to NH_4^+ and CO_2 by urease, extracellular enzyme of microorganisms. Ammonium ion formed due to high activities of urease of our strains caused the *p*H of medium to raise above 9.0 but after 48 hrs of cultivation the *p*H dropped to about 5.0 on account of continuous utilization of ammonium salt and acid formation from glucose. The *p*H of medium should be, therefore, adjusted to constant range of *p*H with alkaline solution in order to keep *p*H of medium from dropping markedly and maintain the favorable environemnt for cell growth. Of various methods adjusting *p*H, the feeding of urea with appropriate intervals is considered to be the most effective for industrial L-glutamic acid fermentation by using our strains in repects of maintaining definite range of *p*H needed for stabilizing the bacterial property and supplying the sufficient nitrogen sources continuously.

3. Natural nutrients for industrial fermentation of L-glutamic acid.

In order to accomplish the possibility of industrial L-glutamic acid fermentation by utilizing our strains, some natural nutrients as shown in Table 10 were examined in this experiment. Resulting from this experiment, some new facts as the followings were confirmed. The extracellular L-glutamic acid (21 mg—23mg/m*l*) more than 40% (w/w) of initial sugar content (5%) could be accumulated even in the medium containing pure amino acid (histidine and cysteine) and vitamins (biotin and thiamine) without natural nutrients, when *Brev. ammoniagenes* T-1 and *Brev. ammoniagenes* YR-2 were cultured. In *Brev. ammoniagenes* Y-2, however, much lower quantities (16.2mg/m*l*) were produced.

In case that yeast extract was solely added to the basal medium (Y medium),

Table 10. Effect of natural substances as vitamin and amino acid on the cell growth and L-glutamic acid production

Strains	Medium	*p*H	mg wet cells/m*l*	G.A. mg/m*l*	Residual sugar (%)
Brev. ammoniagenes T-1	*A+Y	8.2	59.34	9.25	9.40
	A+R	4.5	35.67	26.75	10.27
	A+W	7.8	26.67	20.50	32.71
	A+V	5.7	37.07	21.65	22.27
	V+B	9.2	12.20	6.85	58.81
	V+T	8.1	27.87	15.15	33.31
	V+P	8.3	26.33	15.25	39.15
	V+M	8.4	20.67	14.50	49.95
	Y	8.0	40.67	18.50	2.26
	Y+B	8.2	48.40	18.35	0.00
	Y+T	8.1	42.13	19.11	1.91
	Y+P	9.0	48.00	18.90	1.22
	Y+M	5.9	40.00	22.15	6.62
Brev. ammoniagenes YR-2	A+Y	8.6	55.60	8.15	0.00
	A+R	5.0	42.66	25.00	2.73
	A+W	8.9	22.93	14.80	36.54
	A+V	5.3	28.20	22.15	12.70
	V+B	9.3	7.07	5.50	81.26
	V+T	8.0	29.04	17.90	30.37
	V+M	8.1	24.53	15.25	24.01
	V+P	8.2	30.00	19.00	28.01
	Y	7.9	40.00	18.50	1.74
	Y+B	8.4	52.27	17.30	1.22
	Y+T	8.0	46.00	20.15	1.39
	Y+P	8.2	45.07	18.90	1.74
	Y+M	5.2	48.50	23.65	4.00
Brev. ammoniagenes Y-2	A+Y	8.3	55.26	9.65	1.92
	A+R	6.0	57.00	21.75	3.43
	A+W	5.4	35.47	26.00	3.18
	A+V	8.5	28.13	16.72	26.10
	V+B	9.4	6.70	5.41	79.90
	V+P	9.1	17.33	11.00	58.29
	V+M	9.1	23.80	13.15	25.58
	V+T	8.4	20.87	15.15	20.18
	Y	8.2	39.20	18.90	2.78
	Y+B	8.4	51.47	17.55	1.04
	Y+T	7.9	42.00	20.75	0.00
	Y+P	8.1	39.33	19.30	2.78
	Y+M	5.3	58.80	23.25	6.44

*A: Histidine and cysteine (0.05%)
Y: Yeast extract (0.5%)
R: Rice-bran extract (2m*l*/*l*)
W: Wheat-bran extract (2m*l*/*l*)
V: Biotin (1γ/*l*) and thiamine (1mg/*l*)
B: Beef extract (0.2%)
T: Tryptone (0.2%)
P: Peptone (0.2%)
M: Malt extract (0.2%)
These natural substances were added to the basal medium respectively.

all three strains produced L-glutamic acid in a little lower quantity than in the case of V+A medium but the multiplication of cells was largely increased by 10—40%. When malt extract was added to this Y medium (Y+M medium), there was, compared with in the case of Y medium, about 10—30% increase in L-glutamic acid accumulation by all three strains together with corresponding increase especially in cell growth of *Brev. ammoniagenes* Y-2. When amino acids were added to the Y medium instead of malt extract, however, none of cultures showed increment of L-glutamic acid accumulation but cell populations were strikingly increased by 39—46%. As can be well seen in Table 10, tryptone and peptone were assured to be unsatisfactory nutrients in the presence of yeast extract, and beef extract seemed to act even as an inhibitory substances owing to scarce of amino acids such as histidine and cysteine needed by microorganisms tested.

Brev. ammoniagenes T-1 and *Brev. ammoniagenes* YR-2 showed maximum accumulation of L-glutamic acid when rice-bran extract as a vitamin source was added to the synthetic medium supplemented with histidine and cysteine (A+R medium). The highest yields of L-glutamic acid by *Brev. ammoniagenes* Y-2 was achieved by the

addition of wheat-bran extract (W+R medium). In both cases, L-glutamic acid more than 50% (w/w) of initial sugar content, i.e., 25—27mg of L-glutamic acid per m*l* of medium containing 5% glucose were accumulated.

From all these results, the fact that our strains required vitamin as well as some amino acids for their good growth and L-glutamic acid accumulation was once more recalled. It could be also postulated that all of three strains require an addition to biotin and thiamine, another vitamin substance which seems to be contained in malt extract, rice-bran, and wheat-bran. Of these three expectant vitamin sources, extracts of rice and wheat-bran were found to be much more superior to malt extract in respect of attaining L-glutamic acid fermentation. Yeast extract was not, even though it contains some vitamins and amino acids, effective enough to elevate the L-glutamic acid production rate because of lacking some nutrients essential for L-glutamic acid production.

When both of results obtained from cultures in the Y medium and A+Y medium were compared with each other, it was indicated that amino acid possibly functioned as organic factor needed for only cell growth, far from participating in reaction step of L-glutamic acid production. In a word, it can be said that histidine and cysteine are essential for the growth and multiplication of our strains.

Consequently it must be noted that the remarkably high yields of L-glutamic acid in the A+R and A+W medium indicates the possibility that all three strains may be utilized for industrial fermentation of L-glutamic, if natural resources containing abundant amount of histidine and cysteine be available. For the industrial fermentation of L-glutamic acid, therefore, further researches into natural nutrients resources containing sufficient histidine or cysteine, together with chemical analysis of rice- and wheat-bran extract for detection of elements stimulating the L-glutamic acid synthesis, have to be carried out in the coming day.

Figure 4 and 5 show the relationship between L-glutamic acid production and corresponding cell growth together with the changes of *p*H when each strain was incubated in Y+M and A+V medium. In the A+V medium, the cell populations reached their maximum growths after 12 hrs incubation, whereas L-glutamic acid began to increase markedly between 12 hrs and 24 hrs after incubation. After 24 hrs incubation, L-glutamic acid had increased very slowly along with culture time but began to decrease after 72 hrs. In the Y+M medium, the culture time required for strikingl increment of L-glutamic acid accumulation by *Brev. ammoniagenes* T-1 and *Brev. ammoniagenes* YR-2 was 12hrs later than in the case of V+A medium. In *Brev. ammoniagenes* Y-2, however, it was as same as in the case of V+A medium. As for the cell growth, whereas on the other hand, the same results as above were obtained except for *Brev. ammoniagenes* Y-2, of which cell population reached its peak at 24 hrs after incubation. In any cases, existence of an time interval of about 12 hrs between the time period for maximum formation of L-glutamic acid and for cell growth

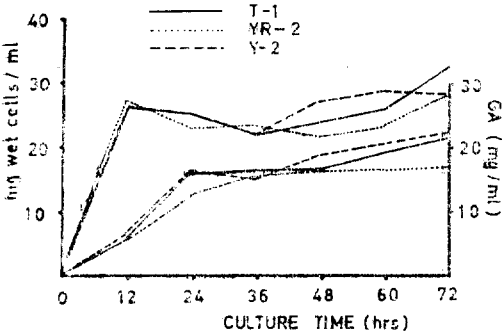

Fig. 4. Relationship between culture time, cell growth and glutamic acid production in the V+A medium

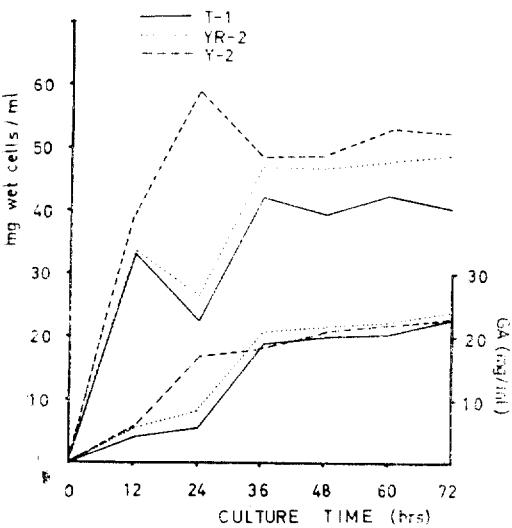

Fig. 5. Relationship between culture time, cell growth and glutamic acid production in the Y+M medium

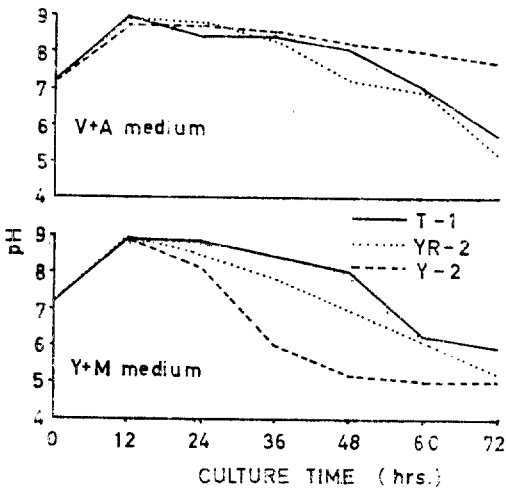

Fig. 6. Changes of *p*H with time course in the A+V and Y+M media

indicated that L-glutamic acid formation began to occur soon after cell multiplication was completed.

In addition, changes of *p*H with time course are illustrated in Figure 6. Contrary to expectation, the *p*H was raised extremely to about 9.0 despite of occurrence of maximum cell growth. This result indicated that there was so much NH_4^+ ions that sufficient NH_4^+ ions enough to maintain the high level of *p*H remained yet.

摘 要

前報에서 分離 報告한 *Brevibacterium ammoniagenes*의 3個 菌株를 利用 各 菌體들의 增殖과 L-glutamic acid 蓄積에 要하는 榮養要求性을 觀察한 바 3個菌株가 모두 特定한 비타민과 아미노산을 同時에 要求함을 確認하였다. 비타민으로서는 biotin과 thiamine 이 함께 요구되는 반면 아미노酸에 의한 菌體의 成長 및 L-glutamic acid의 蓄積은 histidine이나 또는 cysteine에 依하여 거의 같은 程度로 촉진됨을 아울러 確認하였다. 한편 本 菌株들은 rice-bran extract나 wheat-bran extract가 포함된 培地內에서 初糖 50%以上의 L-glutamic acid를 生成함으로서 工業的 利用이 可能함을 보여 주었다.

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