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Studies on the L-Glutamic acid Fermentation(Part II)

L-Glutamic acid Production Employing Enzymatic Hydrolyzate of Tapioca Pellets as Carbon Source

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L-Glutamic acid 醱酵生産에 關한 硏究 (第二報)

Tapioca Pellets 酵素 糖化液을 利用한 L-Glutamic acid 生産

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Abstract

The possibility of using tapioca pellets as a raw material in glutamic acid fermentation by *Micrococcus glutamicus* is shown. The ground pellets were diluted with water to 20% solid level and treated with α-amylase prepared from a thermophilic Actinomycetes strain culture for 90 min at 85°C under pH 6.0. The liquefied solution was further saccharified with commercial glucoamylase for 36 hours under the reaction conditions of 55°C and pH 5.0. The inhibitory effect of excess biotin content, 16 μg per liter of the hydrolyzate, could be reduced effectively by adding 10 IU of penicillin per m*l* of the medium after five hours of the fermentation. The maximum glutamic acid yield of 38.5 g/*l* was obtained after 60 hours of shaking culture at 28-30°C.

I. Introduction

Since the discovery of the monosodium salt of L-Glutamic acid as a seasoning by K. Ikeda in 1908, its demand in food industries has been increased steadily throughout the world. In 1957, Kinoshita et al[1-4] found several bacteria which can produce L-glutamic acid efficiently from glucose. Following this success, the fermentative production of monosodium glutamate on an industrial scale has been expanded rapidly to meet the increasing world need for this product.

In commercial practice, it is imperative that a readily available and inexpensive natural substrate is employed as carbon source for the industrial production of L-glutamic acid. In the fermentative production of L-glutamic acid by using natural resources such as cane or beet molasses[5], the inhibitory effect of excess biotin can be overcome by the addition of antibiotics[6-8], aliphatic alcohols[9-10], fatty acid and its derivatives[11-13],

which enhanced permeability of glutamate.

The present studies were carried out to explore the possibility of evolving a microbial process for glutamate production employing tapioca pellets as a raw material. Tapioca pellets are manufactured from manioc plants [14] (*Manihot esculenta*) cultivated widely in South-East Asia, India, Africa, and South America. It contains large amount of starch of good-quality. Tapioca starch has a bland flavor and forms a clear but cohesive paste. Generally, it is used in foods in the form of partially gelatinized pellets. In addition, it has the lowest gelatinizing temperature[15] among the commonly available starches. Therefore, tapioca pellets are supposed to be easily hydrolyzed into glucose when treated by glycoside hydrolases.

In this paper, it was studied on the conditions for the enzymatic hydrolysis of tapioca starch as well as on the cultural conditions for the fermentative production of L-glutamic acid by using its hydrolyzate as a carbon source.

II. Materials and Methods.

Tapioca starch. Tapioca pellets produced in Thailand were sufficiently ground with a miller (200 mesh) and used as substrate in this work. It was analyzed for the general composition by conventional methods[16-17], as shown in Table 1.

Table 1. General Composition of Tapioca pellets used as Substrate.

Moisture	12.23%
Crude Protein	1.22%
Crude Fat	0.73%
Crude Fiber	7.87%
Nitrogen-free ex.	74.35%
Ash	3.44%

Preparation of α-amylase solution. Thermophilic Actinomycetes strain [18] was inoculated in 30 m*l* of culture medium added with 0.04% of $CaCl_2$ and incubated at 50°C for 16 hr with reciprocal saking (120 stroke/min), The culture medium was consisted of soluble starch 3.0%, peptone 1.0%, yeast extract 0.5%, NaCl 0.5%, $MgSO_4 \cdot 7H_2O$ 0.1%, K_2HPO_4 0.02% and $FeSO_4 \cdot 7H_2O$ 2ppm. The initial pH was adjusted to 7.0. After incubation, the culture medium was centrifuged at 3000 rpm for 10 min and the supernatant was used as crude enzyme solution (3000 DU/m*l*).

α-Amylase activity was determined by a modified method [19] based on blue value method. One DU is defined as the amount of enzyme which is able to reduce 10% of blue value of iodine color in 1min, in acting on 1 m*l* of soluble starch solution being equivalent to 10 μmole of glucose at pH 6.0 at 50°C.

Glucoamylase. A product of Korean Pacific Chem. Industry Co., Ltd., having enzyme activity of 4,050 AU/gr, was used in this work. One AU [20] (Amylolytic Unit) is defined as the amount of enzyme which produces 1 mg of glucose from 5 m*l* of 1% starch solution at pH 5.0 at 40°C in 10 min.

Determination of Liquefaction rate. The degree of liquefaction was determined by Iodine coloration value[21]. One m*l* of the hot filtrate from the liquefied solution was added to 1 m*l* of 2N caustic soda solution. The mixture was heated to 60°C for 1 min. Then, 80m*l* of water, 2 m*l* of 2N hydrochloric acid and 10m*l* of the iodine reagent (0.5% KI, 0.05% I_2) were added, and total volume was adjusted to 100m*l* with distilled water. The aliquot was determined for optical density at 660 mμ. Iodine coloration value was expressed in the optical density value.

Determination of saccharification rate. The conversion rate of carbohydrates to glucose was expressed as dextrose equivalents (DE) which is per cent of reducing sugars based on the weight of total carbohydrates. The ground tapioca pellets were analyzed for total carbohydrates by acid hydrolysis[17] and the reducing sugars detemined by the modified method of Somogyi. [16]

Microorganism. Micrococcus glutamicus (renamed *Corynebacterium glutamicum*) maintained on nutrient agar slant was used for L-glutamic acid fermentation.

Fermentation. The medium[22] employed in L-glutamic acid production is shown in Table 2. The hydrolyzate was diluted to contain 10 per cent of

reducing sugar. The initial pH was adjusted to 7.0 with dilute NaOH solution. Prior to the fermentation, the preculture was carried out as follows; A loopful of the test strain was transferred into 30m*l* of the seed medium composed of glucose 0.5%, urea 0.5% peptone 0.5%, beef extract 1.0%, NaCl 0.5% and incubated at 28°C for 24 hr on a reciprocal shaker. One milliliter of the culture broth was transferred into 50m*l* of the medium for glutamate production in a 500m*l* shaking flask. Fermentation was carried out at 28°C for 72 hr on the reciprocal shaker. 0.5% of Urea as weight per volume was added to the medium at the beginning of the fermentation and equal amount of it was fed 4 times every 12 hr during the fermentation.

Determination of L-glutamic acid. L-Glutamic acid was estimated by paper chromatography method[23], and also by copper complex salt method [24] using spectrophotometric measurement.

Determination of cell growth. The growth of cells was determined by the measurement of the absorbance at 660mμ of cultured broth diluted forty folds.

Determination of biotin[25]. The amount of biotin in the medium for L-glutamic acid production was estimated by microbiological assay using *Lactobacillus arabinosus.*

Table 2. Composition of medium for L-glutamic acid fermentation.

Casamino acid	0.05%
Inorganic salts;	
KH_2PO_4	0.1 %
$MgSO_4 \cdot 7H_2O$	0.04%
Fe^{++}	2 ppm
Mn^{++}	2 ppm
Thiamin-HCl	200 μg
Urea*	2.5%
Hydrolyzate**	1000m*l*
pH	7.0

* 0.5% Urea was added to the medium at the beginning and fed 4 times in the course of cultivation.

** Hydrolyzate was equivalent to 10% Glucose.

III. Results and Discussion

Effect of preheat treatment[21] *on enzyme hydrolysis of tapioca starch.* As the results of preliminary works, it was apparent that the heat treatment was unnecessary as in every case the better yields were obtained without pressure cooking, and that tapioca starch was readily converted into sugars. This might be due to the continued action of the liquefying enzyme[18] which appeared to be quite stable at 65-85°C, and also due to the fact that tapioca starch granules have lower gelatinizing temperature[15] (62.5°C) and are easier in swelling than the other kinds of starch granules.

Effects of concentration and reaction time of α-Amylase on the degree of hydrolysis of tapioca starch. In order to see how the amount of the liquefying enzyme influence sugar yields, 6 g of tapioca pellets was dispersed in 30m*l* of water containing 1 mM $CaCl_2$ and 5 mM NaCl to make a slurry. The pH was adjusted at 6.0 by addition of 50 mM acetate buffer. Then, the various concentrations of α-amylase were added to the slurry. The liquefaction was performed at 75°C, and the rates of liquefaction were determined periodically during the reaction. As shown in Fig. 1, there was no significant difference in the degree of liquefaction when the amylase was added with concentrations above 30 DU per g of the substrate, whereas the liquefaction increased with the enzyme concentration up to this level. Into the liquefied solution cooled to 55°C, glucoamylase was added and it was saccharified for 48 hr to see the effect of liquefaction on the saccharification rate. The best saccharification was observed in the liquefied solution prepared by treating with 30 DU of α-amylase per g of the substrate for 90 min.

Effects of liquefying pH and temperature on the hydrolysis of tapioca starch. In case of liquefaction temperatures, it is considered that higher temperature is desirable for the swelling of associated starch molecules to facilitate the hydrolysis by α-amylase but there is a limit in the heat stability of α-amylase. To examine the optimum pH and temperature ranges for the liquefaction, the

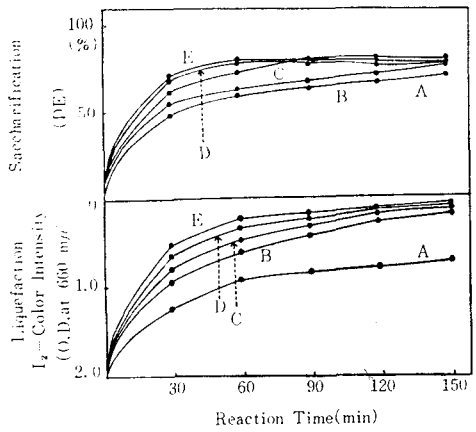

Fig. 1. Effect of α-Amylase Concentration on Liquefaction and Saccharification of Tapioca Starch.

A: 10 DU of α-amylase/g of the substrate added.
B: 20 DU of α-amylase/g of the substrate added.
C: 30 DU of α-amylase/g of the substrate added.
D: 40 DU of α-amylase/g of the substrate added.
E: 50 DU of α-amylase/g of the substrate added.

tapioca slurries containing 30 DU of α-amylase per g of the substrate, 1 mM $CaCl_2$ and 5 mM NaCl, were liquefied under the various pH and temperature ranges for 90 min. After the liquefaction, all these samples were cooled to 55°C and added with glucoamylase to be saccharified in the same manner as before. As indicated in Fig. 2, it was observed that the liquefaction rate at 75±1°C was better than that at the other temperatures, however, the best saccharification was shown in the liquefied solution prepared by treating at 85±1°C. One of the reasons for this result is supposed that in the saccharified liquid a small amount of insoluble starch particles would be produced during the process of liquefaction at lower temperatures than 80°C as described in many reports[26]. The optimum pH for the liquefaction was found to be at 6.0.

Effect of glucoamylase concentration on saccharification rate (DE). To test the optimum concentration of glucoamylase for the saccharification of

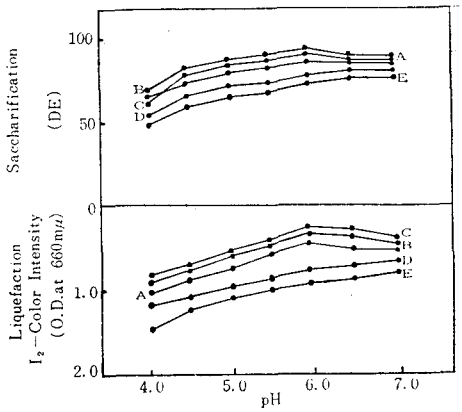

Fig. 2. Effects of Liquefying pH and Temperature on Liquefaction and Saccharification of Tapioca Starch.

A : 95±1°C　　B : 85±1°C
C : 75±1°C　　D : 65±1°C
E : 55±1°C

the liquefied solution prepared from tapioca pellets, the various concentrations of glucoamylase were added to the liquefied solutions obtained under the best liquefying conditions. The saccharification was performed at pH 6.0, at 55°C. As the results of this study presented in Fig. 3, it was apparent

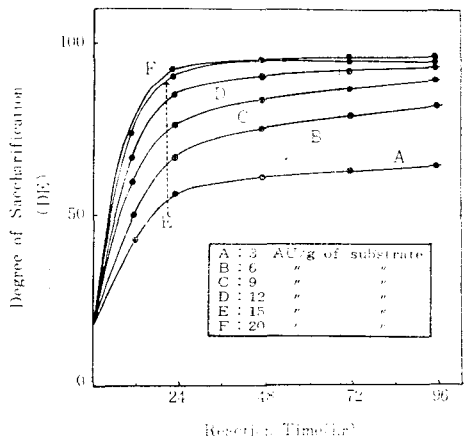

Fig. 3. Effect of Glucoamylase Concentration on Saccharification of Tapioca Starch.

that the highest DE was given in the solution reacted with 15 AU of glucoamylase per g of the substrate for 48 hr. If the reaction was allowed to continue after the highest DE had been reached, the DE began to fall. This effect, which was more noticeable at the higher enzyme concentrations than 15 AU per g of the substrate, might be due to the resynthesis of maltose and isomaltose from glucose. The reaction is catalysed by glucoamylase as described in many reports. [27,28]

Effects of saccharifying pH and temperature on sacharification rate (DE). The liquefied solution containing 15 AU of glucoamylase per g of the substrate was saccharified under the various pH and temperature ranges for 48 hr to see the optimum range for the saccharification. As the data recorded in table 3, the highest DE of 98.3% was shown when the glucoamylase action was conducted at pH 5.0 at 55°C.

Table 3. Effects of Saccharifying pH and Temperature on Saccharification Rate of Tapioca Starch

Temp. (°C)	pH				
	4.5	5.0	5.5	6.0	6.5
50	87.2*	90.4	89.2	89.3	89.1
55	96.3	98.3	97.4	77.3	96.8
60	94.1	97.5	95.6	96.1	96.0
65	86.4	88.5	87.3	85.8	85.1

* DE=reducing sugar/total carbohydrates×100

Effects of saccharification rates (DE) on the production of L-glutamic acid. In case of using the enzyme hydrolyzate of tapioca starch as carbon source in glutamic acid fermentation, it is supposed that the yield of glutamic acid would be partially influenced by the saccharification rate (DE) during the process of the hydrolysis. To test the effect of DE on the glutamic acid production, the fermentation was carried out by using the saccharified samples obtained at periodic intervals during the reaction with glucoamylase.

After the fermentation, the cell density and extracellular glutamate were determined. As the results shown in Fig. 4, it was seen that the highest DE was shown in the solution saccharified for 48 hr, but the accumulation of extracellular glutamic acid and cell growth were given maximum yields in using the hydrolyzate saccharified for 36 hr. As one of the methods to confirm this results, the following experiment was undertaken. The sugars in the hydrolyzate during the saccharification were separated by paper chromatography[29][30] as follows: Descending paper chromatography was carried out on Schlecher & Schnell paper 2043 with the solvent of n-butanol-pyridine-H_2O (9:5:4 v/v)

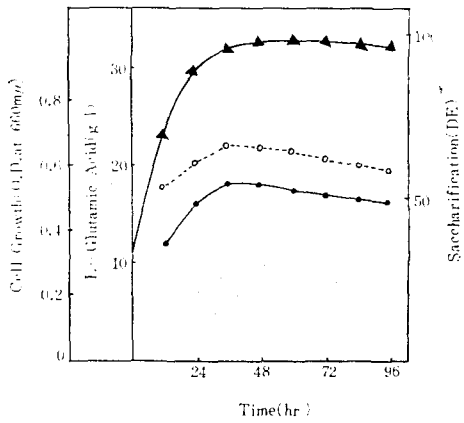

Fig. 4. Effect of Saccharification Time on L-Glutamic Acid Fermentation
▲—▲ : Degree of Saccharification
●—● : L-Glutamic Acid
○···○ : Cell Growth

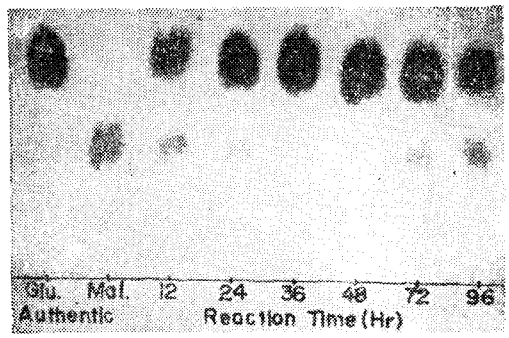

Fig. 5. Paper Chromatograms of the Enzymatic Hydrolyzates from Tapioca Starch.

Table 4. Effect of Hydrochloric acid Concentration, Pressure and Time on the Hydrolysis of Tapioca Starch**

pre. (kg/m²) / T. (min) / HCl (%)	1.5			2.0			2.5		
	10	20	30	10	20	30	10	20	30
0.3	40.4*	42.5	58.5	52.5	58.7	73.5	76.2	78.3	78.5
0.5	61.2	64.2	84.2	85.8	92.4	98.2	90.6	89.4	89.4
0.8	74.5	83.8	91.5	88.4	93.5	98.6	93.1	92.5	90.8
1.0	76.2	87.4	94.3	89.9	93.2	96.7	90.4	89.7	84.3

* DE=reducing sugar/total carbohydrates×100

** 20gr of Tapioca pellets was dispersed in 100ml of water to make it slurry.

for 8 hr. Acetone saturated with $AgNO_3$ and 2 N-NaOH: Ethanol (1:3 v/v) were used to detect the sugars on the paper chromatograms. A reproduction of the chromatographs obtained from these runs is given in Fig. 5.

It was observed that considerable variation in sugar composition could be obtained by the varying saccharification times along with glucoamylase. In the solution saccharified for more than 36 hr, most of the carbohydrates were converted to glucose, and then disaccharide began to be detected again after 72 hr of the saccharification.

It might be due to the resynthesis[27] of maltose from glucose. From these results, it was supposed that the hydrolyzate saccharified for 36 hr was desirable for the glutamate fermentation as a carbon source.

Studies on the acid hydrolysis[2,31] of tapioca starch. In order to see the optimum conditions for the acid hydrolysis of tapioca starch, 20 g of tapioca pellets was dispersed in 100ml of water containing various concentrations of hydrochloric acid and this slurry was tested for the effect of heat treatment and reaction time on the hydrolysis. For the respective cases, the degrees of hydrolysis were determined by the same manner as before. From the results expressed in Table 4, it was apparent that the highest hydrolysis rate, 98.6%, was obtained when the slurry was hydrolyzied with 0.8% of HCl at 2.0kg/cm² for 30 min.

Production of L-glutamic acid using enzyme or acid hydrolyzate as carbon source. From the results to compare the suitability of the enzyme hydrolyzate with the acid as carbon source for the glutamate fermentation, the enzyme hydrolyzate of tapioca starch was preferred because the product obtained in this case was less brownish in colour and the amount of extracellular glutamate in it was better than that in the acid hydrolyzate as well as the growth of the organism. These results are reported in Table 5.

Table 5. Prodution of L-Glutamic Acid using Enzyme and Acid Hydrolyzate* as Carbon Sources

Carbon Sources	Cell Growth (O.D. at 660 mμ)	Extracellular L-GA (g/l)
Enzyme Hydrolyzate	0.648	18.5
Acid Hydrolyzate	0.589	14.8

* Each Hydrolyzate was equivalent to 10% Glucose

Effect of the addition of penicillin[6,8] on L-glutamic acid fermentation. By microbiological assay it was found that the enzyme hydrolyzate of tapioca pellets contained considerable amount of biotin which in the fermentation medium would be so much in excess as to inhibit glutamate excretion by the organism. The biotin content of the enzymatic hydrolyzate after dilution to contain 10 per cent reducing sugar was found to be 16μg/*l*. It has been shown earlier by kinoshita. S.[1], and Okumura, S.[32] that in glucose containing synthetic medium, 2-4μg of biotin per liter of the fermentation broth was optimum for glutamate excretion by *M. glutamicus*. In order to counteract the inhibitory effect of excess biotin on glutamate production, the

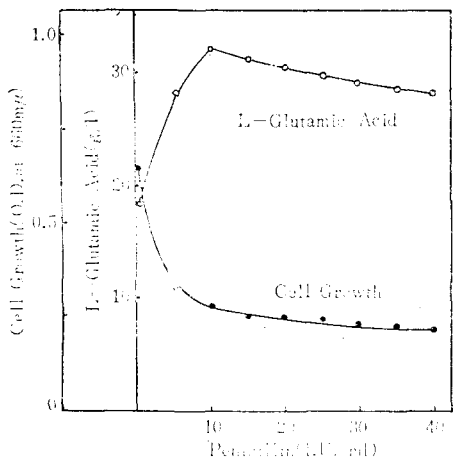

Fig. 6. Effect of Penicillin Concentration on L-Glutamic Acid Fermentation
Various concentrations of penicillin were added at 6 hr after inoculation. The fermentation was carried out at 28°C, for 48 hr with reciprocal shaking.

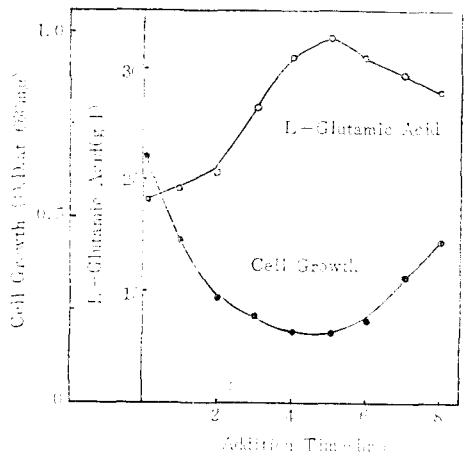

Fig. 7. Effect of Addition Time of Penicillin on L-Glutamic Acid Fermentation.
10I. U. /ml of penicillin was added at various times after inoculation. The fermentation was carried out at 28°C for 48 hr with reciprocal shaking.

addition of penicillin was found to be quite effective. In the experiment, various concentrations of penicillin were added to the growing culture of *M. glutamicus* at various times after fermentation.

Table 6. Effect of Organic Nutrients on L-Glutamic Acid Fermentation

Growth Factor	Conc. of Addition (%)	Cell Growth (O. D. at 660mμ)	L-Glutamic Acid (g/l)
No Addition	0. 0	0. 312	29. 5
Polypeptone	0. 1	0. 325	30. 1
Yeast-extract	0. 1	0. 311	32. 8
Soy bean powder	0. 1	0. 324	32. 3
C. S. L.	0. 5	0. 304	32. 5
Beef-extract	0. 1	0. 298	33. 2
Casamino acid	0. 1	0. 215	33. 4
Gluten-Acid-Hydrolyzate*	1. 0	0. 322	36. 5

* This is manufactured by Korean Saem Pyo Food Industrial Co. and used for the preparation of soy sauce.

As the results shown in Figs 6 and 7, the best effective concentration and addition-time of penicillin on the fermentation in the medium of the enzyme hydrolyzate were obtained when 10 I. U. /ml of penicillin was added at 5 hr after fermentation.

Influences of various organic nutrients on glutamic acid yield. Various organic nutrients as indicated in Table 6 were tested for their suitability to promote glutamate production in the medium of the enzyme hydrolyzate. The compounds were added in quantities which gave almost the same amount of nitrogen in each case. As shown in Table 6 and Fig. 8, it was seen that "Gluten-acid-hydrolyzate" was served as the most suitable nutrient when added to the medium at 1. 5 per cent as volume per volume. It remains unknown what components of "Gluten-acid-hydrolyzate" have an influence on the high yield of glutamate. This problem has been studied in our laboratory and will be the subject of a future report.

Time course of the fermentation. Under the best conditions for the production of glutamate from tapioca pellets which were studied in this report, the fermentation was carried out in the medium supplemented with "Gluten-acid-hydrolyzate" instead of casamino acid, at 28°C with reciprocal shaker. At the periodic intervals during the fermentation, extracellular glutamate, cell

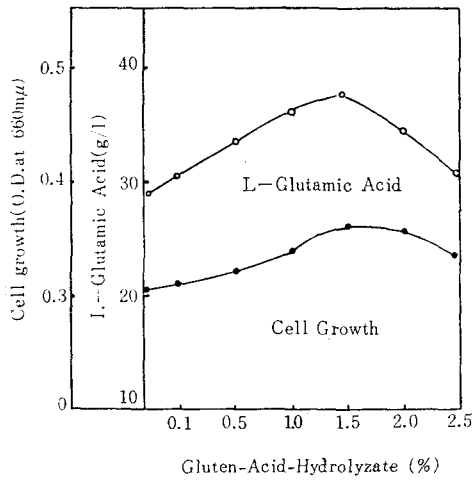

Fig. 8. Effect of Concentration of "Gluten-Acid-Hydrolyzate" on L-Glutamic Acid Fermentation.

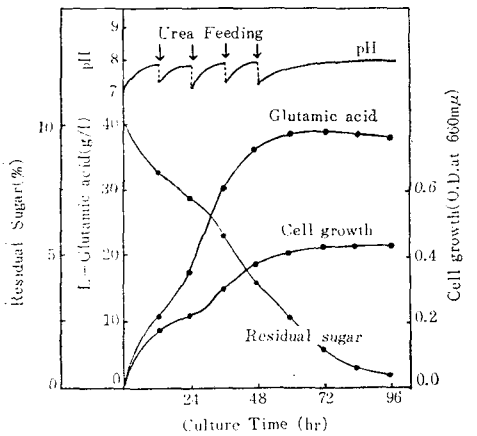

Fig. 9. Time Course of L-Glutamic acid Fermentation

density, pH and residual sugar content of the fermentation broth were determined.

As the results shown in Fig. 9, it was observed that the accumulation of glutamate was rapidly increased after 24 hr of the fermentation and its maximum yield was 38.5 g/*l* after 60 hr of the fermentation. The cell growth was showed a rapid increase after 30 hr and attained a steady state by the 60 th hr. In order to maintain at the optimum range of pH for the *glutamate* production, urea feeding performed during the fermentation.

The results have indicated the possibility of utilizing tapioca pellets as a raw material for glutamic acid fermentation. The large scale trials with aids for aeration, agitation and pH controlling (the time-course study has suggested a possible controlling role of pH) would help in the assessment of the economic feasibility of the process.

IV. 要　約

本 實驗은 *Micrococcus glutamicus* 菌株에 依한 L-Glutamic acid 醱酵 生産에 있어서 Tapioca 澱粉의 酵素糖化液의 利用 可能性을 檢討하였으며, 그 結果는, 20%濃度의 Tapioca 澱粉乳液에 高溫性 放線菌 液化酵素(3000 DU/ml)를 基質 g當 30 DU 添加하고, 85±1°C, pH 6.0에서 90分間 液化한 후, Glucoamylase(4050 AU/g)을 基質 g當 15 AU 添加하고, 55°C, pH 5.0에서 36時間 加水分解시킨 糖化液을 炭素源으로 使用하였을 경우가 L-Glutamic acid 生産量이 가장 良好하였다.

糖化液中의 Biotin 含量은 16 μg/*l* 로서, 過量의 Biotin 濃度로 因한 L-Glutamic acid 生産抑制를 解決하기 위하여 添加된 penicillin 濃度는 培養液ml當 10 I.U.로 培養 5時間後 添加하였을 경우가 가장 良好한 結果를 나타냈으며, *L-Glutamic acid* 生産量은 培養 60時間에서 38.5 g/*l*로. 最大値를 나타냈다.

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