

PATENT SPECIFICATION



NO DRAWINGS

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COMPLETE SPECIFICATION

A method of producing l-glutamic acid by fermentation

We, KYOWA HAKKO KOGYO KABUSHIKI KAISHA, a corporation organised under the laws of Japan, of 9, Yurakucho-1-chome, Chiyoda-ku, Tokyo, Japan, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

- 10 This invention comprises a method of producing l-glutamic acid by fermentation, and more particularly, a method of producing and accumulating l-glutamic acid in a substantial amount by culturing a new strain of Micrococcus in a culturing medium under suitable fermentation conditions.

It is well known that microorganisms in a suitable medium can produce various kinds of amino acids. The amount produced has, however, been extremely small and it has never been reported that a large amount of any particular kind of amino acid could be accumulated in such a medium, by means of a fermentation process.

- 25 One reason which has been suggested to account for the extreme difficulty of accumulating any amino acid in a fermenting medium has been based on the fact that amino acids are components of proteins, and that any amino acid produced in such a medium is apt to be easily converted to a protein or like polypeptide, or is apt to be converted or decomposed to other substances by various biochemical reactions. In other words, it is unlikely that an amino acid can easily be accumulated in a culturing medium in a monomeric state, i.e. a mono-molecular state whether it may be as a free acid or as a salt.

- For this reason, a fermentation process, i.e. the direct use of the biochemical activities of a living population of microorganism, has never been proposed heretofore for the biosynthesis and accumulation of l-glutamic acid. The known biosynthesis is effected in some enzymatic system, that is, a particular

kind of enzyme is extracted from microorganisms, or from some animal or plant tissues, and the enzyme is reacted with suitable substrates e.g. α -ketoglutaric acid and ammonium compounds under very restricted reaction conditions.

An object of the present invention is to provide a method for producing a substantial amount of l-glutamic acid directly by culturing a certain microorganism in a liquid medium.

According to the present invention, a new strain of Micrococcus or an l-glutamic acid-producing mutant thereof is cultured in a suitable medium under adequate fermentation conditions, and a substantial amount of l-glutamic acid as such, is accumulated in the medium.

We have found that a microorganism which satisfies two biochemically specific requirements, that is (1) to have a capacity of producing α -ketoglutaric acid from saccharic materials and (2) to have a strong l-glutamic acid dehydrogenase activity, particularly a strong activity towards the reverse reaction of the above reversible enzymatic reaction, i.e. the reductive amination of α -ketoglutaric acid, is suitable for the direct production of a substantial amount of l-glutamic acid by a fermentation process.

The characteristic feature of the present invention lies in the production and accumulation of l-glutamic acid in the culturing medium by the biochemical activities above mentioned during growth and multiplication of the strain. There is thus an essential difference between the process of the present invention, and the reported enzymatic production wherein l-glutamic acid is synthesized by the reaction of an extracted enzyme and with specific substrates.

A new strain has now been found, which belongs to the genus Micrococcus, and which will be referred to as Micrococcus No. 534. The general characteristics of the strain will be

(Price 3s. 6d.)

described below. Most of the tests were done according to "Manual of Methods for Pure Culture Study of Bacteria". (Society of American Bacteriologists, 9th edition).

5 A. Morphological observations:

I. Microscopical observations:

(Nutrient agar, 37°C., 18 hrs.)

- 10 1. Form: micrococci, occurring singly, in pairs or irregular masses, spherical or slightly ellipsoidal. Size of majority: 0.6—1.2 microns

2. Motility: none

3. Spore: none

- 15 4. Gram stain: positive

II. Agar Stroke:

(Nutrient agar, 37°C., 18 hrs.)

- 20 1. Growth: moderate
2. Form of growth: filiform
3. Lustre: dull
4. Chromogenesis: milky white to slightly yellowish white
5. Odour: absent
6. Consistency: butyrous to slightly viscid
25 7. Colour of medium: unchanged

III. Agar Colonies:

(Nutrient agar, 37°C., 20 hrs.)

- 30 1. Form: circular
2. Surface: smooth
3. Edge: entire
4. Elevation: slightly raised
5. Optical character: opaque

IV. Agar Stab:

35 (Nutrient Agar, 37°C., 20 hrs.)

1. Growth: best at top

V. Nutrient Broth:

(Nutrient Broth, 37°C., 18 hrs.)

- 40 1. Surface growth: ring
2. Clouding: slight
3. Odour: absent
4. Sediment: flocculent, scanty

B. Physiological properties:

- 45 1. Growth temperature:
no growth above 47°C., slight growth around 42°C., good growth between 27—37°C., Optimum around 30°C.
2. Oxygen: strict aerobe
50 3. Nitrate reduction: strong
4. Indol production: absent
5. Hydrogen sulphide: not produced
6. Indicator reduction:
55 methylene blue, 2, 6-dichlorophenolindophenol, yause green, 2, 3, 5-triphenyltetrazoliumchloride are reduced.
7. Gelatin liquefaction: negative or very weak
60 8. Casein digestion: weak
9. Voges-Proskauer reaction: negative
10. Methyl red reaction: slight acidic
11. Citric acid utilization: negative
12. Starch liquefaction: none
65 13. Milk: no change or slightly alkaline

14. Catalase: very weak

15. Urease: strong

16. L-Glutamic acid dehydrogenase activity: very strong

17. Acid production: 70
α-ketoglutaric acid, lactic acid are formed. Other organic acids, trace

18. Carbohydrate fermentation:

	Acid	Gas	
arabinose	±	—	
xylose	±	—	
glucose	+	—	
fructose	+	—	
galactose	±	—	80
mannose	+	—	
lactose	—	—	
dextrin	—	—	
glycerol	—	—	
sucrose	+	—	85
maltose	+	—	
trehalose	—	—	
raffinose	—	—	
melezitose	—	—	
starch	—	—	90
mannitol	—	—	
sorbitol	—	—	
salicin	—	—	
inulin	—	—	
glycogen	—	—	95

(+ indicates presence, — indicates absence and ± indicates doubtful.)

In view of the above properties and observations, it is believed that the present strain belongs to a new species of the genus of 100 *Micrococcus*, because there is none with which it can be identified in Bergey's Manual of Determinative Bacteriology.

Fermentation Procedures:

Medium composition can be any of the 105 known ones suitable for the growth of the *Micrococcus*. As for the source of carbon, any fermentable carbohydrates which will be clear from the aforementioned table and other fermentable carbohydrates, such as hydro- 110 lyzed starch, may be used. As for sources of nitrogen and other nutrients, there are also no specific limitations. To obtain vigorous carbohydrate metabolism of the culture, various kinds of organic or inorganic 115 nitrogenous materials, and various minerals required by the organism should be added to the culturing medium. The culture is inoculated and propagated under aerobic conditions at a suitable temperature, e.g. 120 27—30°C. In the course of the fermentation organic acids tend to accumulate and render the medium acidic, and it has been found that the production of L-glutamic acid is sub- 125 stantially affected by pH value of the medium during the course of the fermentation. The pH value should be maintained at between 6.0 to 9.0, and preferably from 7.0 to 8.5. Thus, the pH value is advantageously ad- 130 justed and maintained within the range from

6.0 to 9.0 by addition of neutralizing agents. Owing to the strong reductive amination activity of the l-glutamic acid dehydrogenase of the present strain, and by keeping the pH value within the correct range, the various unfavourable side-reactions are minimized. Thus the l-glutamic acid formation reaction takes place in preference to the side-reactions, and results in the accumulation of a large amount of l-glutamic acid.

In order to adjust and maintain the pH value of the culture medium within the range from 6—9, a neutralizing agent, such as ammonia, a compound containing a basic nitrogenous radical or a caustic alkali, may be used. Urea is a preferred neutralizing agent since it is converted into ammonia. In any case, ammonium ions, or urea which is converted in the medium into ammonium ions, should be present in the culture medium in order to obtain a high accumulation of l-glutamic acid.

The l-glutamic acid accumulated in the medium is recovered by a known suitable process, such as an ion-exchange resin method, suitable processes being described and claimed in Specification No. 811688.

The following Examples are given for the purpose of illustrating the invention.

Example 1. Shaking culture fermentation in a synthetic medium

Micrococcus No. 534 was cultured for 24 hours in glucose bouillon by shaking cultures at 28°C. and 33°C. and used as inoculum. 30 ml of fermentation medium was distributed in a 250 ml flask and inoculated from the above inoculum and shaken by a rotary shaker of 220 r.p.m. The composition of the fermentation medium was as follows: glucose 50 g, urea 8 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g and $FeCl_3 \cdot 6H_2O$ 5 mg per 1 litre, the pH being 7.5. During the fermentation the pH of the culture medium was adjusted and maintained with a range from 7 to 8.5 by the addition of a 10% urea solution as neutralizing agent.

The analytical results are given in Table 1.

TABLE I

Culturing temperature °C	Culturing duration day	pH	Residual glucose g/100 ml	l-Glutamic acid mg/100 ml	Yield based on consumed glucose %
33	1	8.6	2.8	660	30.0
	2	7.3	1.9	700	22.0
28	2	8.2	2.3	610	22.6
	3	6.8	1.2	810	21.3
	4	8.7	0.2	1130	23.5

Example 2. Shaking culture fermentation in complex medium.

Inoculum and procedures were the same as in Example 1. The fermentation medium was as follows: glucose 100 g, meat extract 5 g, N-Z-amine 5 g, urea 15 g, K_2HPO_4 1 g and $MgSO_4 \cdot 7H_2O$ 0.25 g per 1 litre. Temperature 65 at 28°C.

The analytical results are given in Table 2.

TABLE II

Culturing duration day	pH	Residual sugar g/100 ml	l-Glutamic acid mg/100 ml	Yield based on consumed sugar %
1	8.4	7.2	370	13.6
2	8.2	5.5	1280	28.4
3	7.3	3.5	1960	30.1
4	6.2	2.9	2480	35.2

Various other saccharidic materials, and 75 various other organic and inorganic nitrogenous sources, for example ammonia, ammonium salts such as ammonium chloride, ammonium sulphate, ammonium carbonate and ammonium acetate, peptone, digested 80 soya bean meal, digested fish meal, casein hydrolysate and corn steep liquor are usable in place of the nutrient materials in the fermentation medium mentioned in Example 2.

As will be clear from the above Examples, in a synthetic medium as well as in an organic medium, an amount as high as 20—30% of consumed sugar can be converted to l-glutamic acid.

The new strain which is referred to throughout the specification and the claims as "Micrococcus No. 534" has been designated as Micrococcus glutamicus No. 534 and has been deposited in Washington D.C., U.S.A., 95 with the American Type Culture Collection on the 28th January 1958 and now bears A.T.C.C. No. 13032.

WHAT WE CLAIM IS:

1. A method of producing l-glutamic acid 100 as such, comprising culturing Micrococcus No. 534, or one of its l-glutamic acid-producing mutants, as herein defined, in a culturing medium containing ammonium ions, fermentable sources of carbon, the 105 source of carbon being a saccharidic material, and nitrogen and inorganic materials under aerobic conditions, while maintaining the pH value of the culturing medium within the range of 6 to 9 by the addition of acid- 110 neutralizing agents, whereby a substantial amount of l-glutamic acid is accumulated in the culturing medium.

2. A method according to Claim 1, in which the carbon source is glucose, fructose, 115 sucrose, maltose, xylose and/or hydrolysed starch.

3. A method according to Claim 1 or 2, in which the nitrogen source is urea, ammonia, an ammonium salt, peptone, corn steep 120 liquor, hydrolysed casein, digested fish meal, meat extract and/or digested soya bean meal.

4. A method according to Claim 1, 2 or 3, wherein at least one of the acid-neutralizing agents is a compound containing a basic 125 nitrogenous radical.

5. A method according to Claim 4, wherein at least one of the acid-neutralizing agents is ammonia.

6. A method according to Claim 4 or 5, 130 wherein at least one of the acid-neutralizing

6.0 to 9.0 by addition of neutralizing agents. Owing to the strong reductive amination activity of the l-glutamic acid dehydrogenase of the present strain, and by keeping the pH value within the correct range, the various unfavourable side-reactions are minimized. Thus the l-glutamic acid formation reaction takes place in preference to the side-reactions, and results in the accumulation of a large amount of l-glutamic acid.

In order to adjust and maintain the pH value of the culture medium within the range from 6—9, a neutralizing agent, such as ammonia, a compound containing a basic nitrogenous radical or a caustic alkali, may be used. Urea is a preferred neutralizing agent since it is converted into ammonia. In any case, ammonium ions, or urea which is converted in the medium into ammonium ions, should be present in the culture medium in order to obtain a high accumulation of l-glutamic acid.

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Example 2. Shaking culture fermentation in complex medium.

Inoculum and procedures were the same as in Example 1. The fermentation medium was as follows: glucose 100 g, meat extract 5 g, N-Z-amine 5 g, urea 15 g, K_2HPO_4 1 g and $MgSO_4 \cdot 7H_2O$ 0.25 g per 1 litre. Temperature 55 at 28°C.

The analytical results are given in Table 2.

TABLE II

Culturing duration day	pH	Residual sugar g/100 ml	l-Glutamic acid mg/100 ml	Yield based on consumed sugar %
1	8.4	7.2	370	13.6
2	8.2	5.5	1280	28.4
3	7.3	3.5	1960	30.1
4	6.2	2.9	2480	35.2

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2. A method according to Claim 1, in which the carbon source is glucose, fructose, sucrose, maltose, xylose and/or hydrolysed starch.

3. A method according to Claim 1 or 2, in which the nitrogen source is urea, ammonia, an ammonium salt, peptone, corn steep liquor, hydrolysed casein, digested fish meal, meat extract and/or digested soya bean meal.

4. A method according to Claim 1, 2 or 3, wherein at least one of the acid-neutralizing agents is a compound containing a basic nitrogenous radical.

5. A method according to Claim 4, wherein at least one of the acid-neutralizing agents is ammonia.

6. A method according to Claim 4 or 5, wherein at least one of the acid-neutralizing



agents is urea.

7. A method according to any of Claims 1, 2 or 3, wherein at least one of the acid-neutralizing agents is a caustic alkali.

5 8. A method of producing l-glutamic acid by fermentation substantially as hereinbefore described.

9. A method of producing l-glutamic acid

according to any of the foregoing Examples.

10. l-glutamic acid, whenever prepared by 10 a process according to any of the preceding Claims.

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