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(54) METHOD OF PRODUCING L-GLUTAMIC ACID

(71) We, TAKEDA YAKUHIN KOGYO KABUSHIKI KAISHA (TAKEDA CHEMICAL INDUSTRIES, LTD.), of 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, a corporate body organised under the laws of 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:—

This invention relates to a novel and improved method for producing L-glutamic acid by cultivating microorganisms in a culture medium containing acetic acid.

There have been known a number of microorganisms which are capable of producing and accumulating L-glutamic acid in the culture media. However, those microorganisms which are employed in the industrial production of L-glutamic acid generally require biotin for their growth, and, nevertheless, their ability to produce L-glutamic acid is remarkably suppressed by the presence of biotin in an amount exceeding a certain level in the culture medium. This means that a troublesome procedure is required for adjusting the action of biotin in the culture medium throughout the cultivation. Furthermore, most common and economical nutrient sources such as molasses, starch hydrolysate, cornsteep liquor and yeast extracts often contain too much biotin for carrying out the hitherto known processes by the use of the known microorganisms, unless the action of biotin is suitably adjusted, which has constituted one of drawbacks of the known methods.

It has been known that L-glutamic acid is produced by cultivating microorganisms in a culture medium containing acetic acid. But such known methods are industrially not practical due to the poor yield of L-glutamic acid.

[Price 25p]

We have sought to provide an industrially feasible method for producing L-glutamic acid by cultivating a microorganism which is not affected by the amount of biotin and by the use of inexpensive acetic acid as a carbon source, the method resulting in a good yield and a high purity of L-Glutamic acid.

Throughout this specification the percentages concerning the ingredients in the culture media are based on weight per volume basis, i.e. grams per 100 ml of the medium.

Percentages quoted in other respects are based on weight per weight basis.

This object is realized by cultivating under aerobic conditions an L-Glutamic acid-producing microorganism which belongs to the genus *Brevibacterium*, *Corynebacterium* or *Micrococcus* and which does not require biotin, but requires an unsaturated fatty acid having from 16 to 22 carbon atoms, in a culture medium containing a carbon source, a nitrogen source, a source of the higher unsaturated fatty acid having from 16 to 22 carbon atoms and other necessary nutrient sources, and maintaining the concentration of the carbon source in the culture medium at not more than 1% by the addition of acetic acid during the cultivation period later than the stage at which the original carbon source in the culture medium has been consumed to reach a concentration below 1%.

The microorganisms employable in the method of the present invention are exemplified by *Brevibacterium thiogenitalis* D248, *Brevibacterium thiogenitalis* D253, *Brevibacterium thiogenitalis* D254, *Brevibacterium sp. 111-S09*, *Brevibacterium flavum* BN.11, *Corynebacterium sp. No. 1602*, *Corynebacterium sp. No. 186*, *Micrococcus glutamicus* No. 534-MS-023 and *Micrococcus glutamicus* No. 541-MS-117.

The unsaturated fatty acid having from 16 to 22 carbon atoms is exemplified by oleic acid, palmitoleic acid, linoleic acid, linolenic acid, arachidonic acid or a mixture of any of these fatty acids. It is to be noted that the unsaturated fatty acid source used in the method of this invention includes, for example, the unsaturated fatty acids, their salts (e.g. sodium, potassium or ammonium salt) and their polyhydric alcohol esters such as glycerides (e.g. lard or olive oil) or ethylene glycol esters. It is generally enough to simply add such an unsaturated fatty acid source in an amount of 50 to 1000 γ /ml. to the culture medium.

The carbon source employable in the original culture medium may be any of those which are conventional and is exemplified by molasses and starch hydrolysate as well as acetic acid.

The nitrogen sources employable in the method of the present invention are exemplified by cornsteep liquor, defatted soybean powder, ammonia and urea.

Other nutrients employable in the method of the present invention are exemplified by sodium phosphate, potassium phosphate, calcium chloride and magnesium sulphate.

In the method of the present invention, it is completely unnecessary to control the biotin action as is required in various known methods since the result of the present invention is not affected by the amount of biotin in the culture medium.

In the method of the present invention, the acetic acid as the carbon source may be added in the form of salts such as sodium, potassium, ammonium or calcium salts instead of free acetic acid. When acetic acid is used as an initial carbon source, its concentration in the culture medium is desirably not higher than 2% by weight relative to the volume of the initial culture medium. However after the stage at which the original carbon source content has been reduced as low as 1% or less, production of L-glutamic acid is carried out by controlling the acetic acid concentration in the medium to a value not higher than 1%.

In order to control the acetic acid concentration in the culture medium it is preferable to check the amount of the dissolved oxygen in the culture medium, and provided this is not higher than about 5 ppm at a pH of from about 7 to about 8, the acetic acid concentration is well adjusted to be in the desirable range. The adjustment of the pH is conveniently made by the addition of a base such as

aqueous ammonia or urea, which may be used as a part of the nitrogen source.

As is understood from the above explanation, acetic acid may be used as a sole main carbon source throughout the cultivation, and it is noteworthy that highly pure crystals of L-glutamic acid are obtainable when acetic acid is used as the sole main carbon source.

L-glutamic acid thus produced in the culture medium is recovered in conventional manner, for example, by filtering the culture broth, adjusting the pH of the resulting filtrate to 3.2 by the addition of hydrochloric acid, and collecting the precipitate of L-glutamic acid therefrom.

The following examples are given to illustrate the invention. The abbreviation "IFO" denotes "Institute for Fermentation Osaka".

EXAMPLE 1

A seed culture medium containing glucose (2.0%), urea (0.5%), KH_2PO_4 (0.1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), cornsteep liquor (0.5%) and sodium oleate (100 γ /ml. as oleic acid) is poured, in 20 ml. portions into 200 ml. Erlenmeyer flasks, and these flasks are sterilised. One of the nine strains listed in the following table is inoculated in the medium and incubated at 28°C. on a 200 r.p.m. rotary shaker for 24 hours.

A main culture medium containing an enzymic hydrolysate of sweet potato starch 5% as direct reducing sugar, molasses (5% as sugar), urea (1.5%), KH_2PO_4 (0.1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), cornsteep liquor (0.5%) and sodium oleate (300 γ /ml. as oleic acid) is poured in 70 ml. portions, into 200 ml. conical flasks, and is sterilised. 3.5 ml. portions of the seed culture are inoculated into the main culture medium, and the cultivation is carried out at 30°C. on 200 r.p.m. rotary shaker. At the 24th hour of the incubation, urea is added in an amount of 0.5% relatively to the culture broth. About 30 hours after the start of the incubation, that is, when the residual sugar content in the culture medium has reached a concentration of 1% or less, fractional feed of acetic acid is started so as to maintain the dissolved oxygen concentration in the medium at about 5 ppm or less, while aqueous ammonia is added dropwise so as to keep the culture medium at a pH of 7.0 to 7.3. The cultivation is completed in 48 hours. The results are summarised in the following table.

Microorganism	Amount of L-glutamic acid before feed of acetic acid (mg./ml.)	Final amount of L-glutamic acid (mg./ml.)	Total amount of acetic acid fed (%)	Yield of L-glutamic acid to acetic acid fed (%)
<i>Brevibacterium thiogenitalis</i> D-248 IFO-12331	52	66	2.5	58
<i>Brevibacterium</i> sp. 111-809 IFO-12332	45	53	1.8	44
<i>Brevibacterium thiogenitalis</i> D-253 IFO-12400	46	55	2.0	46
<i>Brevibacterium thiogenitalis</i> D-254 IFO-12401	47	54	1.6	44
<i>Corynebacterium</i> sp. No. 1602 IFO-12399	50	62	2.3	53
<i>Corynebacterium</i> sp. No. 186 IFO-12398	51	65	2.5	55
<i>Micrococcus glutamicus</i> No.534-MS-023 IFO-12523	48	57	2.0	46
<i>Micrococcus glutamicus</i> No. 541-MS-117 IFO-12524	50	60	2.2	48
<i>Brevibacterium flavum</i> BN-11 IFO-12525	49	59	2.1	46

EXAMPLE 2

Brevibacterium thiogenitalis D-248 IFO-12331, cultivated in a Sakaguchi's flask, is inoculated in 30 l. of a sterilised seed culture medium of the same composition as the seed culture medium of Example 1, and is cultivated at 28°C. for 16 hours. 5 l. of the above-prepared seed culture are inoculated in 100 l. of a sterilised main culture medium containing molasses (5% as sugars), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), urea (0.1%), corn-steep liquor (0.3%), KH_2PO_4 (0.2%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%) and sodium oleate (200 γ /ml. as oleic acid), and is cultivated at 30°C. under agitation at a rate of 150 r.p.m. with aeration of 25 l. per minute for 48 hours. When the residual sugar content in the culture medium becomes 1.0%, continuous feeding of a 50% aqueous solution of acetic acid to the culture medium is started so that the oxygen concentration in the culture medium may be kept in a range of 0 to 2 ppm. The cultivation is carried out for 48 hours, throughout which time an aqueous solution of ammonia is added to the culture solution so that the pH of the culture solution may be automatically kept within a range of 7 to 7.3.

The bioassay of the resulting culture broth, employing *Lactobacillus arabinosus*, shows

that 60 mg./ml. of L-glutamic acid is produced. The yield of the L-glutamic acid is about 46.5% relatively to the acetic acid consumed.

The resulting culture broth is filtered, and the filtrate is adjusted to pH 3.2 by the addition of hydrochloric acid to separate crystals. The crystals are collected to obtain 7.1 kg. of L-glutamic acid.

EXAMPLE 3

A seed culture medium containing glucose (2.0%), urea (0.5%), KH_2PO_4 (0.1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), cornsteep liquor (0.5%) and sodium oleate (100 γ /ml. as oleic acid) is poured in 20 ml. portions, into 200 ml. Erlenmeyer flasks, and is sterilised. One of the ten microorganisms listed in the following table is inoculated in the culture medium and is cultivated at 28°C. on a 200 r.p.m. rotary shaker for 24 hours.

A main culture medium containing ammonium acetate (1.2%), cornsteep liquor (0.3%), KH_2PO_4 (0.1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (0.001%), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01%), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005%), sodium oleate (200 γ /ml. as oleic acid), vitamin B₁ (100 γ /ml. as hydrochloride) and phenol red (10 mg./ml.) is poured in 70 ml portions into 200

5 ml. fluted flasks, and is sterilised. 3.5 ml. portions of the above-prepared seed culture are inoculated into the main culture medium, and the cultivation is carried out at 32°C. on a 200 r.p.m. rotary shaker.

For checking the acetic acid concentration in the culture medium, a dissolved oxygen analyser is provided for each flask at the start of the cultivation. When the amount of the dissolved oxygen in the culture medium becomes higher than 5 ppm (this means that the major part of the acetic acid has been consumed), glacial acetic acid is started to be

added to the culture medium so as to make its concentration about 0.1% relative to the volume of the culture medium, while the pH of the culture medium is adjusted to from 7 to 8 by the addition of aqueous ammonia according to the indication of the colour change of phenol red. The cultivation is stopped, as illustrated in the table, when the acetic acid has been consumed in an proportion of about 15% relative to the volume of the initial culture medium. The results are shown in the following table.

Microorganism	Cultivation period (hours)	Yield of L-glutamic acid to the acetic acid fed (%)
<i>Brevibacterium thioenitalis</i> D-248 IFO-12331	45	52
<i>Brevibacterium</i> sp. 111-SO9 IFO-12332	51	45
<i>Brevibacterium thioenitalis</i> D-253 IFO-12400	43	50
<i>Brevibacterium thioenitalis</i> D-254 IFO-12401	43	48
<i>Corynebacterium</i> sp. No. 1602 IFO-12399	48	45
<i>Corynebacterium</i> sp. No. 186 IFO-12398	42	53
<i>Micrococcus glutamicus</i> No. 534-MS-023 IFO-12523	43	41
<i>Micrococcus glutamicus</i> No. 541-MS-117 IFO-12524	45	48
<i>Brevibacterium flavum</i> BN-11 IFO-12525	50	45
<i>Brevibacterium flavum</i> ATCC 14067	43	31

Remark: *Brevibacterium flavum* ATCC 14067 requires biotin for its growth and is employed as a control.

EXAMPLE 4

Brevibacterium thioenitalis D-248, IFO-12331, cultivated in a Sakaguchi's flask, is inoculated in 30 l. of a sterilised seed culture medium having the same composition as the seed culture of Example 3, put in a 50 l. fermentor, and cultivated at 28°C. for 16 hours as described in Example 3. 5 l. of the above-prepared seed culture are inoculated in 100 l. of a sterilised main culture medium containing ammonium acetate (1.2%), corn-steep liquor (0.1%), KH_2PO_4 (0.1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01%), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.01%), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (0.001%) and sodium oleate (250 γ /ml. as oleic acid), thiamine hydrochloride (100 γ /l.) in a 200 l. fermentor, and are cultivated at 32°C. with aeration of 30 l. per minute under agitation at a rate of 240 r.p.m. When the dissolved oxygen concentration in the culture medium becomes 5 ppm or higher, a sterilized 40%

aqueous solution of glacial acetic acid is continuously fed to the culture medium so as to make the acetic acid concentration about 0.1% relative to the volume of the initial culture medium. The cultivation is carried out for 48 hours while the pH of the culture medium is adjusted to 7.0 to 8.0 by the addition of aqueous ammonia to the culture medium, whereby 15% of acetic acid relative to the volume of the initial culture medium is consumed.

Bioassay of the resulting culture broth in the same manner as in Example 3 shows that 48 mg./ml. of L-glutamic acid are produced. The yield of the L-glutamic acid relative to the acetic acid consumed is 49%. the resulting culture broth is filtered, and the pH of the filtrate is adjusted to about 3.2 by the addition of hydrochloric acid to separate precipitates, which are collected and crystallised to yield 6.6 kg. of L-glutamic acid.

WHAT WE CLAIM IS:—

1. A method of producing L-glutamic acid, which comprises cultivating under aerobic conditions an L-glutamic acid-producing microorganism, which belongs to the genus *Brevibacterium*, *Corynebacterium* or *Micrococcus* and which does not require biotin but requires an unsaturated fatty acid of 16 to 22 carbon atoms, in a culture medium containing a carbon source, a nitrogen source, a source of the unsaturated fatty acid of 16 to 22 carbon atoms and other nutrient sources necessary for its growth, maintaining the concentration of the carbon source at not more than 1% by the addition of acetic acid during the cultivation period later than the stage at which the original carbon source in the culture medium has been consumed to reach a concentration below 1%; and recovering the L-glutamic acid produced and accumulated in the culture medium.

2. A method as claimed in claim 1, wherein the microorganism is *Brevibacterium thio- genitalis* D248 IFO-12331, *Brevibacterium thio- genitalis* D253 IFO-12400, *Brevibacterium thio- genitalis* D254 IFO-12401, *Brevibacterium* sp. 1111-SO9 IFO-12332, *Brevibacterium flavum* BN.11 IFO-12525, *Corynebacterium* sp. No. 1602 IFO-12399, *Corynebacterium* sp. No. 186 IFO-12398, *Micrococcus glutamicus* No. 534-MS-023 IFO-12523 and *Micrococcus glutamicus* No. 541-MS-1117 IFO-12524.

3. A method as claimed in claim 1 or 2, wherein oleic acid, palmitoleic acid, linoleic acid, linolenic acid, arachidonic acid or a mixture thereof is used as the unsaturated fatty acid.

4. A method as claimed in any of claims 1 to 3, wherein the unsaturated fatty acid is added to the culture medium in an amount of from 50 to 1000 γ /ml of the culture medium.

5. A method as claimed in any of claims 1 to 4, wherein the original carbon source is acetic acid.

6. A method as claimed in claim 5, wherein the amount of the acetic acid is not higher than 2% relative to the volume of the initial culture medium.

7. A method as claimed in claim 1 substantially as herein described with reference to any of the specific examples.

8. L-glutamic acid when prepared by a method as claimed in any of claims 1 to 7.

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Reference has been directed in pursuance of Section 9, Subsection (1) of the Patents Act, 1949, to patents Nos: 1,176,150 and 1,183,235.

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