

PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

Method for Producing L-Isoleucine by Fermentation Process

We, KYOWA HAKKO KOGYO COMPANY LIMITED, a Corporation organised under the laws of Japan of 4, Ohtemachi-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:—

The present invention relates to the production of L-isoleucine by fermentation.

L-isoleucine is an essential amino acid and work has been done on its production by fermentation. The following L-isoleucine-producing bacteria are known: *Bacillus subtilis* No. 14 (Amino Acids 1, 89, (1959), published by the Symposium of Amino Acids Fermentation of Japan), *Bacillus subtilis* No. 14—2, and No. 14—545, *Serratia marcescens*, *Erwinia carotovora*, *Aerobacter aerogenes*, *Pseudomonas aureofaciens* (Ibid. 2, 100, (1960)), *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (Ibid. 2, 90 (1960)).

As a result of further work on various amino acid fermentations, and especially on L-isoleucine fermentation, it has now been found possible to isolate microorganisms which produce and accumulate a remarkable amount of L-isoleucine in a nutrient medium

and so provide commercial methods of producing L-isoleucine.

According to the present invention there is provided a process for producing L-isoleucine which comprises culturing aerobically *Bacillus megatherium*, *Bacillus licheni*, *Bacillus licheniformis*, *Pseudomonas ovalis*, *Brevibacterium ammoniagenes*, *Achromobacter pentosus*, *Paracolobactrium aerogenoides*, *Escherichia freundii*, *Escherichia coli*, *Streptomyces flaveolus* or *Micrococcus glutamicus*, or a mutant strain thereof at 24°—37°C. in a culture medium which comprises a source of assimilable carbon, a source of assimilable nitrogen, inorganic salts and α -aminobutyric acid and separating the L-isoleucine produced.

Of the microorganisms used in the method of the invention, the bacteriological properties of *Micrococcus glutamicus* have been reported in Specification No. 839,597 and the Bulletin of the Agricultural Chemical Society of Japan 22, 176, (1958). The term, "mutant strains" as used herein means strains which can be produced by treating the microorganisms to cause a mutation, for example by irradiation with ultraviolet rays, X-rays or γ -rays, or by treatment with chemical reagent. For instance, the microorganisms listed below are mutant strains and have a powerful L-isoleucine producing ability.

Micrococcus glutamicus 534—Co 146 (requiring methionine)

ATCC No. 14308

534—Co 147 (requiring threonine)

ATCC No. 14296

615—430 (requiring threonine)

ATCC No. 14312

534—106 (requiring leucine)

ATCC No. 14310

534—112 (" ")

ATCC No. 14309

613—211 (" ")

ATCC No. 14311

[Price 4s. 6d.]

Either a synthetic or a complex culture medium may be employed in the present invention. Carbohydrates, such as glucose, fructose, mannose, galactose, sucrose, maltose, glycerol, mannitol, starch hydrolyzate and molasses, as well as organic acids, may be employed as a source of assimilable carbon. As a source of assimilable nitrogen, ammonia, various inorganic and organic ammonium salts, such as ammonium sulphate, chloride, nitrate, phosphate, carbonate and acetate, various nitrates, urea, and other nitrogen-containing materials, such as peptone, NZ-amine, meat extract, yeast extract, corn steep liquor, and the hydrolyzates of various proteinaceous materials such as casein, fish meal, soy bean meal, defatted, dried silk-worm pupae, and fermentation residues may be employed.

As the source of inorganic salts, potassium monohydrogen phosphate, potassium dihydrogen phosphate, magnesium sulphate, calcium carbonate and ferric chloride may be employed.

When the microorganism employed is a mutant strain it is necessary that an adequate amount of any specific growth-promoting substance required by the particular microorganism is present in the culture medium. Particularly when using a synthetic medium, an amount of the necessary growth promoting substance, or a material containing it, has to be added. When using a complex medium containing natural materials, however, the specific addition of such a substance is not necessary, since the natural materials generally contain the necessary growth promoting agents in sufficient amount. 0.5 to 3% of α -aminobutyric acid is preferably added to the medium, to ensure the production of an accumulated amount of L-isoleucine, the former being the biosynthetic precursor of the latter.

The ingredients of the medium may be added all at once at the start of the cultivation, or they may be added continuously or intermittently during the course of the cultivation.

The cultivation is carried out under an aerobic condition, for example, by shaking or by stirring with aeration. The best cultivation temperature varies depending upon the kind of bacteria employed, but it is always in the range of 24° and 37°C. The period of cultivation is generally 2 to 7 days, during which period L-isoleucine is accumulated in the cultivation liquor.

At the end of the cultivation, the cultivation liquor is filtered, and the filtrate is treated according to any of the known processes for the separation of L-isoleucine. For instance, the filtrate is passed through an ion exchange resin to adsorb L-isoleucine, the resin is eluted and the eluate concentrated. After cooling the concentrate crude crystal-

line L-isoleucine separates and may be recrystallized, if necessary, to give purified crystalline L-isoleucine.

The following Examples illustrate the invention. Percentage values are in weight per volume (gms./100 ccs.)

EXAMPLE 1

One loop of the cells of *Brevibacterium ammoniagenes* (B-7-8) (ATCC No 6871) taken from an agar slant medium is inoculated into 10 ml. of a seed culture medium containing 1% of glucose, 1% of peptone, 1% of meat extract, and 0.5% of sodium chloride and having the pH of 7.0, and cultivated at 30°C. for 24 hours on a test tube shaker. A 0.3 ml. portion of the resulting fermented liquor is seeded to 6 ml. of a fermentation medium comprising 10% of glucose, 2% of ammonium sulphate, 0.6% of peptone, 1% of α -aminobutyric acid, 2.5% of calcium carbonate, 10 γ /litre of biotin, 0.1% of potassium monohydrogen phosphate, and 0.03% of magnesium sulphate (heptahydrate), and cultivated at 30°C. for 96 hours with shaking.

The resulting liquor contains 8.82 mg./ml. of L-isoleucine. There is little by-production of other amino acids in the liquor, except a small amount of α -aminobutyric acid.

EXAMPLE 2

Proceeding as in Example 1 *Micrococcus glutamicus* strain 615-430 (ATCC No. 14312) (a threonine-requiring mutant strain) is cultured in a fermentation medium containing 10% of glucose, 2% of ammonium sulphate, 2.5% of calcium carbonate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 1.5% of NZ-amine, 10 γ /litre of biotin, and 1.0% of α -aminobutyric acid (pH 7.0). After 96 hours, the cultivation liquor contains 10.7 mg./ml. of L-isoleucine.

EXAMPLE 3

Proceeding as in Example 1, *Micrococcus glutamicus* strain 534-106 (ATCC No. 14310) (a leucine-requiring mutant strain) is cultured in a fermentation medium containing 10% of glucose, 2% of ammonium sulphate, 2.5% of calcium carbonate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 10 γ /litre of biotin, 1% of α -aminobutyric acid, and 300 γ /ml. of L-leucine (pH 8.0). After 96 hours, the cultivation liquor contains 8.55 mg./ml. of L-isoleucine.

When the amount of α -aminobutyric acid in the fermentation medium of the above-mentioned process, is varied to 1.5%, the amount of L-isoleucine produced in the cultivation liquor is 9.7 mg./ml.

EXAMPLE 4

Proceeding as in Example 1, *Micrococcus*



glutamicus strain 534—Co—147 (ATCC No. 14296) (a threonine-requiring mutant strain, is cultured in a fermentation medium containing 5% of cane sugar, 1.5% of ammonium sulphate, 2% of calcium carbonate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 1% of α -aminobutyric acid, 7.5 γ /litre of biotin, and 400 γ /ml. of L-threonine. After 96 hours, the cultivation liquor contains 7.0 mg./ml. of L-isoleucine.

EXAMPLE 5

Proceeding as in Example 1, *Escherichia coli* strain 15 (ATCC No. 9723) is cultured in a fermentation medium containing 7.5% of glucose, 1.0% of NZ-amine, 1.5 to 2.0% of ammonium sulphate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 2% of calcium carbonate, and 1% of α -aminobutyric acid (pH 7.0—7.5). After 96 hours, the cultivation liquor contains 7.5 mg./ml. of L-isoleucine with only a small amount of contaminating amino acids.

EXAMPLE 6

Proceeding as in Example 1, a *Streptomyces flaveolus* strain is cultured in a fermentation medium containing 5% of glucose, 1% of ammonium chloride, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 13 mg./litre of ferrous sulphate, 1% of calcium carbonate, and 0.6% of α -aminobutyric acid (pH 7.0). After 96 hours, the cultivation liquor contains 3 mg./ml. of L-isoleucine.

EXAMPLE 7

Three litres of a fermentation medium containing 10% of glucose, 2% of ammonium sulphate, 2.5% of calcium carbonate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 1.5% of NZ-amine, 10 γ /litre of biotin, and 1.5% of α -aminobutyric acid (pH 7), are placed in a 5 litre fermentation tank, and sterilized. After cooling, a cultivated seed of *Micrococcus glutamicus* strain 615—430 (ATCC No. 14312) is inoculated into the medium. After the beginning of the cultivation, 0.7% glucose solution is supplied three times every 24 hours, and the cultivation is carried out at 30°C. for 5 days under stirring with aeration. The amount of L-isoleucine produced in the cultivation liquor is 14.5 mg./ml.

The three litres of the cultivation liquor are filtered, and the filtrate is passed through a cation-exchange resin (Diaion SK No. 1, produced by Mitsubishi Chemical Co. Ltd.

to adsorb L-isoleucine onto the resin. After elution of the resin, the eluate is concentrated. After cooling the concentrate, 41 g. of crude crystalline L-isoleucine are obtained.

EXAMPLE 8

Micrococcus glutamicus No. 534—Co 146 (ATCC No. 14308) (a methionine-requiring mutant strain) is inoculated into a medium containing 10% of glucose, 2% of ammonium sulphate, 2.5% of calcium carbonate, 0.1% of potassium dihydrogenphosphate, 0.03% of magnesium sulphate (heptahydrate), 10 γ /L. of biotin, 0.8% of α -aminobutyric acid and 400 γ /ml. of D,L-methionine. Cultivation is carried out as described in Example 1 and a concentration of 4.2 mg./ml. of L-isoleucine is reached after 96 hours cultivation.

EXAMPLE 9

Micrococcus glutamicus No. 534—112 (ATCC No. 14309) (a leucine-requiring mutant strain) is inoculated into the medium described in Example 3, and cultivation is carried out also as in Example 3. The concentration of L-isoleucine after 96 hours cultivation is 7.61 mg./ml.

EXAMPLE 10

Micrococcus glutamicus No. 613—211 (ATCC No. 14311) (a leucine-requiring mutant strain) is inoculated into the medium as described in Example 3, and cultivation is carried out also as in Example 3. The concentration of L-isoleucine after 96 hours cultivation is 6.35 mg./ml.

WHAT WE CLAIM IS:—

1. Process for producing L-isoleucine which comprises culturing aerobically *Bacillus megatherium*, *Bacillus licheni*, *Bacillus licheniformis*, *Pseudomonas ovalis*, *Brevibacterium ammoniagenes*, *Achromobacter pentosus*, *Paracolonobacterium aerogenoides*, *Escherichia freundii*, *Escherichia coli*, *Streptomyces flaveolus* or *Micrococcus glutamicus*, or a mutant strain thereof at 24°—37°C. in a culture medium which comprises a source of assimilable carbon, a source of assimilable nitrogen, inorganic salts and α -aminobutyric acid and separating the L-isoleucine produced.
2. Process according to claim 1 wherein a mutant strain of *Micrococcus glutamicus* is cultured.
3. Process according to claim 1 or 2 wherein the amount of α -aminobutyric acid added to the medium is 0.5 to 3 weight for volume %.
4. Process for producing L-isoleucine according to claim 1 substantially as described in the Examples.



glutamicus strain 534—Co—147 (ATCC No. 14296) (a threonine-requiring mutant strain, is cultured in a fermentation medium containing 5% of cane sugar, 1.5% of ammonium sulphate, 2% of calcium carbonate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 1% of α -aminobutyric acid, 7.5 γ /litre of biotin, and 400 γ /ml. of L-threonine. After 96 hours, the cultivation liquor contains 7.0 mg./ml. of L-isoleucine.

EXAMPLE 5

Proceeding as in Example 1, *Escherichia coli* strain 15 (ATCC No. 9723) is cultured in a fermentation medium containing 7.5% of glucose, 1.0% of NZ-amine, 1.5 to 2.0% of ammonium sulphate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 2% of calcium carbonate, and 1% of α -aminobutyric acid (pH 7.0—7.5). After 96 hours, the cultivation liquor contains 7.5 mg./ml. of L-isoleucine with only a small amount of contaminating amino acids.

EXAMPLE 6

Proceeding as in Example 1, a *Streptomyces flaveolus* strain is cultured in a fermentation medium containing 5% of glucose, 1% of ammonium chloride, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 13 mg./litre of ferrous sulphate, 1% of calcium carbonate, and 0.6% of α -aminobutyric acid (pH 7.0). After 96 hours, the cultivation liquor contains 3 mg./ml. of L-isoleucine.

EXAMPLE 7

Three litres of a fermentation medium containing 10% of glucose, 2% of ammonium sulphate, 2.5% of calcium carbonate, 0.1% of potassium monohydrogen phosphate, 0.03% of magnesium sulphate (heptahydrate), 1.5% of NZ-amine, 10 γ /litre of biotin, and 1.5% of α -aminobutyric acid (pH 7), are placed in a 5 litre fermentation tank, and sterilized. After cooling, a cultivated seed of *Micrococcus glutamicus* strain 615—430 (ATCC No. 14312) is inoculated into the medium. After the beginning of the cultivation, 0.7% glucose solution is supplied three times every 24 hours, and the cultivation is carried out at 30°C. for 5 days under stirring with aeration. The amount of L-isoleucine produced in the cultivation liquor is 14.5 mg./ml.

The three litres of the cultivation liquor are filtered, and the filtrate is passed through a cation-exchange resin (Diaion SK No. 1, produced by Mitsubishi Chemical Co. Ltd.

to adsorb L-isoleucine onto the resin. After elution of the resin, the eluate is concentrated. After cooling the concentrate, 41 g. of crude crystalline L-isoleucine are obtained.

EXAMPLE 8

Micrococcus glutamicus No. 534—Co 146 (ATCC No. 14308) (a methionine-requiring mutant strain) is inoculated into a medium containing 10% of glucose, 2% of ammonium sulphate, 2.5% of calcium carbonate, 0.1% of potassium dihydrogenphosphate, 0.03% of magnesium sulphate (heptahydrate), 10 γ /L. of biotin, 0.8% of α -aminobutyric acid and 400 γ /ml. of D,L-methionine. Cultivation is carried out as described in Example 1 and a concentration of 4.2 mg./ml. of L-isoleucine is reached after 96 hours cultivation.

EXAMPLE 9

Micrococcus glutamicus No. 534—112 (ATCC No. 14309) (a leucine-requiring mutant strain) is inoculated into the medium described in Example 3, and cultivation is carried out also as in Example 3. The concentration of L-isoleucine after 96 hours cultivation is 7.61 mg./ml.

EXAMPLE 10

Micrococcus glutamicus No. 613—211 (ATCC No. 14311) (a leucine-requiring mutant strain) is inoculated into the medium as described in Example 3, and cultivation is carried out also as in Example 3. The concentration of L-isoleucine after 96 hours cultivation is 6.35 mg./ml.

WHAT WE CLAIM IS:—

1. Process for producing L-isoleucine which comprises culturing aerobically *Bacillus megatherium*, *Bacillus licheni*, *Bacillus licheniformis*, *Pseudomonas ovalis*, *Brevibacterium ammoniagenes*, *Achromobacter pentosus*, *Paracolobactrum aerogenoides*, *Escherichia freundii*, *Escherichia coli*, *Streptomyces flaveolus* or *Micrococcus glutamicus*, or a mutant strain thereof at 24°—37°C. in a culture medium which comprises a source of assimilable carbon, a source of assimilable nitrogen, inorganic salts and α -aminobutyric acid and separating the L-isoleucine produced.

2. Process according to claim 1 wherein a mutant strain of *Micrococcus glutamicus* is cultured.

3. Process according to claim 1 or 2 wherein the amount of α -aminobutyric acid added to the medium is 0.5 to 3 weight for volume %.

4. Process for producing L-isoleucine according to claim 1 substantially as described in the Examples.



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5. L-Isoleucine when obtained by the process claimed in any one of claims 1 to 4.

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