

PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

A method of producing L-Threonine by Fermentation

We, KYOWA HAKKO KOGYO CO. LTD., a corporation organized 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:—

This invention relates to the production of L-threonine by fermentation.

L-threonine is an essential amino-acid for animal and microbial nutrition, and when it has been produced by cultivating a micro-organism in a nutrient medium, it is in general used for the biosynthesis of isoleucine or as a material for constructing protein. Accordingly, it is difficult to accumulate a substantial quantity of L-threonine by culturing a micro-organism in a nutrient medium. It is also known that the enzymatic system which biosynthesises L-threonine from L-homoserine is disturbed by the presence of various amino-acids (Archives Biochemica, Biophysica 78, 416, 1958). The only process so far devised for the production of L-threonine from L-homoserine by direct fermentation using micro-organisms is one using a mutant of *Bacillus subtilis* (Amino acids, No. 1, 74, 1959).

It has now been found that L-threonine may be produced biochemically by the aerobic fermentation of nutrient media containing L-homoserine at 24—37°C. and a pH of 4—7 using, as microorganisms, either (i) *Brevibacterium linens*, *Brevibacterium vitrumen*, *Brevibacterium liticum*, *Micrococcus rubens*, *Micrococcus varians*, *Xanthomonas pruni*, *Sarcina lutea*, or *Corynebacterium rathayi*, in which case the L-homoserine is added to the medium from 6 to 48 hours after the beginning of the culture, or (ii) *Micrococcus glutamicus*, *Vibrio percolans*, *Xanthomonas citri*, or *Corynebacterium simplex*, in which case the L-homoserine may be initially present in the culture medium or may be added during fermentation. In both cases it is preferred to add

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the L-homoserine to the nutrient medium in a number of portions, preferably, when a microorganism of the second group is used, from 6 to 60 hours after the beginning of the culture, and in association with a source of assimilable carbon.

As the nutrient media used in the new process, either organic or synthetic media may be used so long as they contain the necessary nutrients, e.g. sources of assimilable carbon, nitrogen source, and inorganic substances.

As the carbon source, various kinds of carbohydrate materials such as glucose, fructose, sucrose, maltose, glycerol, mannitol, sorbitol, hydrolysate of starch, and waste molasses may be used. These carbon sources may be added to the medium in one lot at the beginning of the culture or in portions during the culture. Organic acids, such as gluconic acid, 2-keto-gluconic acid, fumaric acid, citric acid, lactic acid, malic acid, itaconic acid, aconitic acid, succinic acid, and pyruvic acid may be used, alone or together with a carbohydrate material.

The nitrogen source may be, for example, ammonia, ammonium sulphate, ammonium chloride, ammonium phosphate, a nitrate, urea and an other nitrogen-containing organic substance, such as peptone, NZ-amine, meat extract, yeast extract, corn steep liquor, ~~casein~~, fish meal, soy bean, or a residue of fermentation or a hydrolysate thereof.

The inorganic substances may be for example, potassium dihydrogen phosphate, potassium hydrogen phosphate, magnesium sulphate, and calcium carbonate.

The L-homoserine to be added to the medium may be added as such, i.e. as chemically pure L-homoserine, or in the form of a material containing L-homoserine, for instance an L-homoserine-containing culture solution obtained by a culturing a microorganism or a concentrate thereof. The amount added is preferably about 2 to 20 mg. of L-homoserine per 1 ml. of the nutrient medium.

The fermentation is carried out under aro-

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- bic conditions with shaking or aeration and agitation. A culture temperature of about 30°C. is preferred. During cultivation, the pH of the medium tends to increase to more than 7, but is maintained between 4 and 7 by using a neutralizing agent. Suitable neutralizing agents are mineral acids such as hydrochloric acid or sulphuric acid, or an organic acid. If the pH of the medium rises above 7, the L-threonine produced is decomposed.
- After completion of the culture, L-threonine is recovered from the culture medium by the method set forth in Example 2 below or by a known procedure.
- The following Examples illustrate the invention.

EXAMPLE 1

A culture medium (of pH 8.0) containing, weight for volume, 10% of glucose, 2% of

ammonium sulphate, 0.5% of yeast extract, 0.2% of peptone, 0.1% of potassium dihydrogen phosphate and 0.03% of magnesium sulphate was sterilized, and the pH dropped during the sterilization to 6. 2% of calcium carbonate which had been sterilized separately by dry-heat was then added. The microorganisms shown in Table 1 were inoculated to separate portions of this medium and cultured under aerobic conditions at 30°C., two portions being used for each microorganism. L-homoserine was added to the medium either at the beginning or after 24 hours of the culture, in an amount corresponding to a concentration in the medium of 5 mg./ml., and the medium was then cultured for a further 48 hours. After completion of the culture, the L-threonine accumulated was determined by microbiological assay, and the results are shown in Table 1.

TABLE 1

Microorganism used	Amount of L-threonine in mg./ml. in culture medium when the L-homoserine was added	
	at the beginning of the culture	24 hours after the beginning of the culture
<i>Brevibacterium linens</i>	0.2	1.8
„ <i>vitrumen</i>	0.3	1.6
„ <i>liticum</i>	0.56	1.0
<i>Micrococcus glutamicus</i>	1.2	2.4
„ <i>rubens</i>	0.2	1.1
„ <i>varians</i>	0.18	1.3
<i>Vibrio percolans</i>	1.0	2.0
<i>Xanthomonas citri</i>	0.9	2.8
„ <i>pruni</i>	0.2	2.4
<i>Sarcina lutea</i>	0.3	0.7
<i>Corynebacterium rathayi</i>	0.3	1.1
„ <i>simplex</i>	1.5	1.8

EXAMPLE 2

- Vibrio percolans* was inoculated to the fermentation medium of Example 1, and cultivated under aerobic conditions at 30°C. L-homoserine was added to the medium after 24 hours cultivation in a concentration corresponding to 5 mg./ml. in the medium, and the culture was continued for 48 hours.

After completion of the culture, the medium was filtered to remove mycelium. 1 litre of the filtrate (containing 4.6 mg./ml. of L-threonine) was adjusted to a pH of 2.0 by adding 6N-hydrochloric acid. This solution was fed to a resin column packed with strongly acidic sulphonic acid-type cation exchange resin, which had previously been converted to the

H-form by washing with 2-N hydrochloric acid, and the L-threonine was absorbed on the resin. After the resin had been washed with water, the L-threonine was eluted with aqueous ammonia (3.5%), and the fraction giving a positive ninhydrin-reaction was collected.

Excess ammonia was removed from the eluate by concentration under reduced pressure at a temperature below 50°C., and the solution was then further concentrated after decolorization with active carbon. Ethyl alcohol was added to the resultant concentrated solution, and white powdery L-threonine crystallized, and was separated by filtration and dried. 4 g. of a first crop of crude crystalline L-threonine were obtained. The purity of this

crystal was 91% as determined by Microbiological assay.

EXAMPLE 3

To the same fermentation medium as described in Example 1, *Xanthomonas citri* was inoculated and was cultured under aerobic conditions, L-homoserine was added to different portions of the culture medium 0, 6, 12, 18, 24, 32, and 48 hours after the beginning of the culture in a concentration corresponding to 4 mg./ml. in the medium and the culture was continued for another 48 hours. After completion of the culture, the amount of accumulated L-threonine, as determined by Microbiological assay, was as indicated in Table 2.

TABLE 2

Time of addition (hours)	Accumulated amount of L-threonine (mg./ml.)
0	0.2
6	1.5
12	2.0
18	2.0
24	2.8
32	3.1
48	0.9

It is clear from this Table that the best time for adding the L-homoserine is 6 or more hours after the beginning of the culture.

EXAMPLE 4

To the same fermentation medium as described in Example 1, *Xanthomonas citri* was inoculated. Beginning after 24 hours culture, L-homoserine was added to the medium 4 times separately at intervals of 12 hours, in amount corresponding to a total concentration of 2.5 mg./ml. The amount of accumulated L-threonine in the medium 96 hours after the beginning of the culture was 6 mg./ml., determined by Microbiological assay. On the other hand, when 2% of glucose was added simultaneously with each addition of L-homoserine, the corresponding final concentration of L-threonine was 8.5 mg./ml.

EXAMPLE 5

A material containing L-homoserine was prepared by concentrating a fermentation solution containing 50 mg./ml. of L-homoserine, which had been obtained by culturing an L-

threonine requiring strain of *Micrococcus glutamicus* (ATCC No. 14296).

To the same fermentation medium as described in Example 1, *Xanthomonas citri* was inoculated and cultured under aerobic conditions at 30°C. After 24 hours cultivation, the said material containing L-homoserine was added to the medium so that the concentration of L-homoserine became 5 mg./ml. and the culture was continued for 48 hours more. After completion of the culture, the accumulated amount of L-threonine was 3 mg./ml., determined by Microbiological assay.

WHAT WE CLAIM IS:—

1. Process for producing L-threonine which comprises culturing aerobically *Brevibacterium liticum*, *Micrococcus rubens*, *Micrococcus varians*, *Xanthomonas pruni*, *Sarcina lutea*, or *Corynebacterium rathayi*, in a nutrient medium at 24—37°C. and a pH of 4—7, adding L-homoserine to the medium from 6 to 48 hours after the beginning of the culture continuing the culture, and isolating L-threonine from the culture medium.

2. Process for producing L-threonine which



H-form by washing with 2-N hydrochloric acid, and the L-threonine was absorbed on the resin. After the resin had been washed with water, the L-threonine was eluted with aqueous ammonia (3.5%), and the fraction giving a positive ninhydrin-reaction was collected.

Excess ammonia was removed from the eluate by concentration under reduced pressure at a temperature below 50°C., and the solution was then further concentrated after decolorization with active carbon. Ethyl alcohol was added to the resultant concentrated solution, and white powdery L-threonine crystallized, and was separated by filtration and dried. 4 g. of a first crop of crude crystalline L-threonine were obtained. The purity of this

crystal was 91% as determined by Microbiological assay.

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To the same fermentation medium as described in Example 1, *Xanthomonas citri* was inoculated and was cultured under aerobic conditions, L-homoserine was added to different portions of the culture medium 0, 6, 12, 18, 24, 32, and 48 hours after the beginning of the culture in a concentration corresponding to 4 mg./ml. in the medium and the culture was continued for another 48 hours. After completion of the culture, the amount of accumulated L-threonine, as determined by Microbiological assay, was as indicated in Table 2.

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To the same fermentation medium as described in Example 1, *Xanthomonas citri* was inoculated. Beginning after 24 hours culture, L-homoserine was added to the medium 4 times separately at intervals of 12 hours, in amount corresponding to a total concentration of 2.5 mg./ml. The amount of accumulated L-threonine in the medium 96 hours after the beginning of the culture was 6 mg./ml., determined by Microbiological assay. On the other hand, when 2% of glucose was added simultaneously with each addition of L-homoserine, the corresponding final concentration of L-threonine was 8.5 mg./ml.

EXAMPLE 5

A material containing L-homoserine was prepared by concentrating a fermentation solution containing 50 mg./ml. of L-homoserine, which had been obtained by culturing an L-

threonine requiring strain of *Micrococcus glutamicus* (ATCC No. 14296).

To the same fermentation medium as described in Example 1, *Xanthomonas citri* was inoculated and cultured under aerobic conditions at 30°C. After 24 hours cultivation, the said material containing L-homoserine was added to the medium so that the concentration of L-homoserine became 5 mg./ml. and the culture was continued for 48 hours more. After completion of the culture, the accumulated amount of L-threonine was 3 mg./ml., determined by Microbiological assay.

WHAT WE CLAIM IS:—

1. Process for producing L-threonine which comprises culturing aerobically *Brevibacterium liticum*, *Micrococcus rubens*, *Micrococcus varians*, *Xanthomonas pruni*, *Sarcina lutea*, or *Corynebacterium rathayi*, in a nutrient medium at 24–37°C. and a pH of 4–7, adding L-homoserine to the medium from 6 to 48 hours after the beginning of the culture continuing the culture, and isolating L-threonine from the culture medium.

2. Process for producing L-threonine which



- comprises culturing aerobically *Micrococcus glutamicus*, *Vibrio percolans*, *Xanthomas citri*, or *Corynebacterium simplex* in a nutrient medium containing L-homoserine at 24—37° C. and a pH of 4—7, and isolating L-threonine from the culture medium.
- 5 3. Process as claimed in claim 2 wherein the L-homoserine is added to the culture medium from 6 to 60 hours after the beginning
- 10 of the culture.
4. Process as claimed in claim 3 wherein a source of assimilable carbon is added with the L-homoserine.
- 15 5. Process as claimed in any of claims 1 to 4 wherein the L-homoserine is added to the nutrient medium in a number of portions.
6. Process as claimed in any of the preceding claims wherein the culture was carried out at 30°C.
7. Process as claimed in any of the preceding claims wherein the L-homoserine is added to the nutrient medium in total amount corresponding to a concentration of 2—20 mg./ml.
- 20 8. Process as claimed in claim 1 or 2 substantially as described in the Examples.
- 25 9. L-threonine when produced by the process claimed in any of the preceding claims.

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