

PATENT SPECIFICATION

NO DRAWINGS

922,359



Date of Application and filing Complete Specification: May 30, 1961.

No. 19486/61.

Application made in Japan (No. 27737) on June 15, 1960.

Complete Specification Published March 27, 1963.

Index at acceptance:—Class 2(3), C3A8(A4:B1:C7:D1:K).

International Classification:—C12d.

COMPLETE SPECIFICATION

A Method for Producing Orotic Acid by a Fermentation Process

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 a method for producing orotic acid by a fermentation process.

Orotic acid is a precursor of pyrimidine materials which are components of nucleic acid, and is regarded as identical with vitamin B₁₂, which is important in the metabolism of nucleic acid, multiplication of cells in the living body, protein synthesis, and metabolism of saccharide and ~~liquid~~ substances. It has been found from clinical research on orotic acid that it is remarkably effective in the treatment of liver disease, and that it has a blood-making effect as well as a growth-promoting effect. Thus, orotic acid is becoming of increasing importance.

There have been a large number of reports of research on ~~nucleic~~ acid metabolism using microorganisms from the standpoint of enzymatic chemistry and heredity, and it has become known that mutant strains of *Neurospora crassa*, which require pyrimidines, accumulate a small amount of orotic acid in the culture medium. (Journal of Biological Chemistry 172, 525, [1948]). However, no process for the production of orotic acid by fermentation on a commercial scale has been described.

A full investigation has been carried out on microorganisms which can directly produce orotic acid in a higher yield in a culture liquor using, as main raw materials, a cheap carbon source such as saccharine materials, organic acids and the like and an inorganic nitrogen source. It has been discovered that pyrimidine-requiring mutant strains of organisms having the ability to produce and accumulate amino acids in a medium contain-

ing saccharine materials and inorganic nitrogenous compounds, as main raw materials, have a ability to produce and accumulate orotic acid. By cultivating these microorganisms under suitable culturing conditions orotic acid, as substantially the sole product, is accumulated in a remarkably large amount, with only small amounts of by-products such as amino acid, organic acids and the like. The amount of orotic acid produced is so large that it exceeds by far the solubility of orotic acid, its ~~ammonia~~ salt or other salts in the fermented medium. Thus, a major part of the orotic acid or its salt separates in the crystalline form in the culture medium during the fermentation, and the orotic acid produced can be separated and recovered in a simple manner after the termination of the cultivation.

According to the present invention therefore there is provided a process for producing orotic acid by fermentation which comprises cultivating an orotic acid-producing bacterium selected from pyrimidine-requiring strains belonging to microorganisms of the *Bacillus*, *Brevibacterium*, *Corynebacterium*, and *Micrococcus* groups, in an approximately neutral medium containing a source of carbon, a source of nitrogen, inorganic salts and a pyrimidine and recovering from the fermentation medium the orotic acid produced.

A preferred bacterium for use in the present invention is *Micrococcus glutamici* No. 534—615—305 strain (on deposit at the American Type Culture Collection under accession number ATCC 14275), which needs uracil for its growth an artificial mutant of *Micrococcus glutamici* No. 534 strain. Bacteriological characteristics of *Micrococcus glutamici* have been described in Japanese Patent No. 243,382 and the Bulletin of the Agricultural Chemical Society of Japan, 22, No. 3, pages 176 to 185. The No. 534—615—305 strain was obtained by applying ultra-violet irradiation to the original strain,

[Price 4s. 6d.]



50



55

ammonium

60



65



70

75

Micrococcus

80

glutamici

85



90

been
221



and differs from the latter in needing uracil for growth.

It has been found that in bacteria belonging to *Micrococcus glutamicus*, mutants which require pyrimidines, such as uracil, cytidine, uridine and the like, have the ability to produce a remarkably large amount of orotic acid. It has also been found that pyrimidine-requiring mutants of microorganisms belonging to the *Brevibacterium*, *Bacillus* and *Corynebacterium*, or *Micrococcus* groups, and having ability to produce glutamic acid, also have ability to produce a remarkably large amount of orotic acid.

Any organic or synthetic culture medium may be employed in the present fermentation process, provided that it contains a carbon source, a nitrogen source, inorganic salts and a pyrimidine.

As the carbon source, various carbohydrate materials, such as glucose, fructose, mannose, galactose, sucrose, maltose, mannitol, starch hydrolysate, molasses, and the like, may be used. Also, organic acids, such as gluconic, acetic, lactic, pyruvic, citric, aconitic, α -ketoglutaric, fumaric, malic, oxalacetic, and other acids, may be employed alone or in combination with the carbohydrate material.

As the nitrogen source, ammonia; various inorganic and organic ammonium salts, such as ammonium sulphate, chloride, nitrate, phosphate, carbonate, acetate, and the like; various nitrates; urea, and other nitrogenous compounds, as well as peptone, meat extract, yeast extract, corn steep liquor, amino acids, such as glutamic and aspartic acid, and hydrolysates of protein materials, such as casein, fish meal, soy bean cake, silk-worm chrysalis, and distillation waste residue from alcohol fermentation broth may be used. When a pure synthetic culture medium is used, addition of a proper amount of biotin and uracil, required for growth of bacteria permits accumulation of orotic acid in the same degree or greater degree, as compared with that when using natural organic nutrient source as mentioned above.

Suitable inorganic salts are, for example, dipotassium phosphate, monopotassium phosphate, magnesium sulphate, iron sulphate, manganese sulphate and calcium carbonate.

The fermentation is preferably carried out under aerobic conditions such as, by cultivation under shaking or cultivation under agitation and aeration. The cultivation temperature may be 24° to 37° C., preferably 28° to 33° C. The pH may tend to decrease to below 7.0 during the cultivation, but is then preferably adjusted within 5.5 and 8.5 by addition of a neutralizing agent at the commencement of, or during, the cultivation. Suitable neutralizing agents are, for example, aqueous ammonia or ammonia gas; a caustic alkali such as sodium hydroxide, potassium hydroxide and the like; ammonium carbonate, calcium carbonate; or calcium hydroxide. Addition of urea serves as a supply of ammonia and for the adjustment of pH, which is effective for production of orotic acid. A remarkably large amount of orotic acid accumulates in the culture medium within a cultivation period of 2 to 3 days.

The following examples will serve to illustrate the invention:—

EXAMPLE I

In each of a plurality of 250 ml volume Erlenmeyer flasks, 20 ml of an aqueous fermentation medium is placed, and sterilized prior to use. The medium contained 5.0% of glucose, 1.5% of ammonium acetate, 0.05% of monopotassium phosphate, 0.05% of dipotassium phosphate, 0.5% of urea, 0.5% of yeast extract (which contains a pyrimidine), 0.05% of magnesium sulphate, 0.001% of manganese sulphate, 0.5% of ammonium sulphate and 10 γ /litre of biotin.

Mutants of bacteria listed below, which require a pyrimidine, are inoculated to each of the media, and cultured at 30° C. under shaking. Seventy-two hours later, the amounts of orotic acid produced are estimated, as follows:—

	Name of bacterium	Amount of orotic acid produced (g./100 ml.)
95	<i>Bacillus megaterium</i> - - - - -	0.21
	<i>Bacillus lentus</i> - - - - -	0.28
	<i>Bacillus circulans</i> - - - - -	0.32
	<i>Micrococcus glutamicus</i> No. 534—615—	
	305 (ATCC 14275) - - - - -	0.52
100	<i>Micrococcus glutamicus</i> No. 588—208	
	(ATCC 14276) - - - - -	0.41
	<i>Brevibacterium ammoniagenes</i> ATCC	
	6871 - - - - -	0.30
	<i>Corynebacterium rathayi</i> - - - - -	0.25

EXAMPLE II

The strain employed in the example was *Micrococcus glutamicus* No. 534—615—305. A five hundred ml portion of an aqueous

culture medium is placed in each of a plurality of 2000 ml volume Erlenmeyer flasks, and sterilized prior to use.

The fermentation medium has the follow-



megaterium
95



50

55

60

65

70

75

80

85

90

110



K.H. 3
1/ml, 5
ing composition: glucose, 5.0%; K_2PO_4 , 0.05%; K_2HPO_4 , 0.05%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.001%; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001%; $(\text{NH}_4)_2\text{SO}_4$, 0.5%; urea, 0.5%; biotin, 25 γ /litre; uracil 100 μg /ml, (pH 7.0).

After the inoculation of said strain, the inoculated medium is cultured with shaking while keeping the temperature at 30° C. During the cultivation, urea is added to supply the ammonia source and to adjust the pH of the fermentation liquor to between 6.0 and 8.0. In the course of the cultivation, crystals of ammonia orotate formed in the medium. After 72 hours cultivation, a fermented liquor containing 0.8% of orotic acid is obtained.

EXAMPLE III

The process described in Example II was repeated using a culture medium containing 10.0% of glucose, 0.05% of KH_2PO_4 ; 0.05% of K_2HPO_4 ; 0.05% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.001% of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.001% of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5% of $(\text{NH}_4)_2\text{SO}_4$; 0.5% of urea, 20 γ /litre of biotin, and 100 γ /ml of uracil. The cultivation was continued for 72 hours. The amount of orotic acid produced was 10mg/ml. The crystals which crystallize during the cultivation were isolated, and the filtered fermentation liquor was concentrated. The crystals formed upon cooling of the concentrate were filtered. Thus, ammonium orotate corresponding to 9.2 g. of orotic acid was obtained from 1 litre of the fermented liquor (containing 10 g. of orotic acid). The salt of orotic acid is easily changed to the free acid by heating with dilute hydrochloric acid.

WHAT WE CLAIM IS:—

1. A method of producing orotic acid by

fermentation which comprises cultivating an orotic acid-producing bacterium selected from pyrimidine-requiring strains belonging to microorganisms of the *Bacillus*, *Brevibacterium*, *Corynebacterium*, and *Micrococcus* groups, in an approximately neutral medium containing a source of carbon, a source of nitrogen, inorganic salts and a pyrimidine and recovering from the fermentation medium the orotic acid produced.

2. A method according to Claim 1, wherein the orotic acid-producing bacterium is *Micrococcus glutamicus* No. 534—615—305.

3. A method according to Claim 1, wherein the orotic acid-producing bacterium is *Micrococcus glutamicus* No. 588—208 (ATCC 14276).

4. A method according to any of Claims 1 to 3, wherein biotin is present in the culture medium.

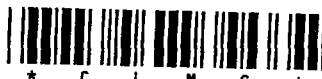
5. A method according to any of Claims 1 to 4, wherein the pH value of the culture medium is maintained from 5.5 to 8.5.

6. A method according to Claim 5, wherein the pH value is maintained by adding one or more agents selected from aqueous ammonia, ammonia gas, sodium hydroxide, potassium hydroxide, ammonium carbonate, calcium carbonate, calcium hydroxide and urea.

7. A method for producing orotic acid according to Claim 1 substantially as hereinbefore described.

8. Orotic acid obtained by a process claimed in any one of the preceding claims.

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ing composition: glucose, 5.0%; K_2PO_4 , 0.05%; K_2HPO_4 , 0.05%; $MgSO_4 \cdot 7H_2O$, 0.05%; $MnSO_4 \cdot 4H_2O$, 0.001%; $FeSO_4 \cdot 7H_2O$, 0.001%; $(NH_4)_2SO_4$, 0.5%; urea, 0.5%; biotin, 25 γ /litre; uracil 100 γ /ml, (pH 7.0).

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3. A method according to Claim 1, wherein the orotic acid-producing bacterium is *Micrococcus glutamicus* No. 588—208 (ATCC 14276).

4. A method according to any of Claims 1 to 3, wherein biotin is present in the culture medium.

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