

PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

A Method for Manufacturing Xanthosine by Fermentation

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:—

10 THIS INVENTION relates to a method for producing xanthosine by fermentation.

Xanthosine is an important material for the biosynthesis of nucleic acid and its 5'-phosphoric acid ester is reported as having a remarkable seasoning action. Xanthosine may be synthesised chemically, but the process is so complicated and the yield is so poor that the method is not suitable for industrial purposes. Consequently, it has hitherto been prepared exclusively by the deamination of guanosine obtained from the decomposition of yeast nucleic acid. It has now been found that when a mutant strain of guanine-requiring *Aerobacter aerogenes* is cultured in a medium under specific culture conditions, xanthosine accumulates in the culture medium.

According to the present invention therefore there is provided a process for producing xanthosine which comprises cultivating at 20° to 40° C. and a pH of 5 to 8 a guanine-requiring mutant strain of *Aerobacter aerogenes* in a nutrient medium comprising assimilable sources of carbon, nitrogen and inorganic salts and guanine, guanosine or guanylic acid in an amount less than that required for the maximum growth of the microorganism and separating xanthosine from the culture medium.

A guanine-requiring strain of *Aerobacter aerogenes* can be obtained by subjecting a wild strain of *Aerobacter aerogenes* to irradiation by ultraviolet light or the X-rays emitted by Co⁶⁰, or by other known muta-

genic treatments and by selecting with penicillin. The guanine-requiring mutant strain thus obtained accumulates xanthosine in a culture medium. Mutant strains which are obtained by performing the mutagenic treatment 2 or more times and which require other growth promoting agents in addition to guanine can also accumulate xanthosine in a culture medium. Consequently, the guanine-requiring mutant strain of the present invention comprises not only a mutant strain requiring merely guanine for its growth, but also a strain which requires, in addition to guanine, other growth promoting agents. The preferred mutant strain of *Aerobacter aerogenes* is *Aerobacter aerogenes* No. 5301 (ATCC No. 14304). Maltose, lactose, sucrose, glucose, fructose and mannitol are the preferred sources of assimilable carbon in the culture medium. The concentration of assimilable carbon is generally about 5%, (gms. per 100 ccs.) higher concentrations may be employed with good results.

As the source of assimilable nitrogen it is possible to utilize ammonia, various inorganic and organic ammonia salts such as ammonium sulphate, ammonium chloride, ammonium nitrate, ammonium phosphate, ammonium carbonate and ammonium acetate. Nitrates, urea, peptones, NZ-amine, meat extract, yeast extract, corn-steep-liquor and the hydrolytic decomposition products of proteinaceous material such as casein, fishmeal, bean-meal, pupae and fermentation residues. Ammonium sulphate, ammonium chloride, ammonium nitrate and ammonium phosphate are preferred as sources of assimilable nitrogen.

As inorganic salts, potassium dihydrogen phosphate, potassium monohydrogen phosphate, magnesium sulphate and calcium carbonate may be utilized. As is well known, microorganisms require certain kinds of metallic ions, especially such metallic ions as

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potassium and magnesium, for their growth, and these ions are usually added to the culture medium in the form of an appropriate soluble salt.

5 Various other metallic ions such as iron, manganese, cobalt and nickel, the required amounts of which are minute, may be added separately if necessary or their presence ensured by using tap water in the preparation of the culture medium.

10 Furthermore, the additional growth promoting agents necessary for certain of the mutant strains should be added to the culture medium. The microorganisms employed in the present invention are mutant strains which require guanine for their growth, so that guanine, guanosine or guanylic acid, their nontoxic salts, such as the hydrochloride, or materials contain such compounds, for example yeast extract, fish extract, the acid hydrolysate of microorganisms and yeast hydrolysate should be added to the culture medium. Substantially no xanthosine is produced when more guanine is added than is required for the maximum growth of the mutant strain. When the amount of guanine added is less than the amount required for maximum growth of the mutant strain there is a remarkable accumulation of xanthosine.

15 The amount of guanine required in the culture medium for maximum growth depends on the concentration of the assimilable carbon source in the medium, normally, when the concentration of carbon source is from 1—10% (weight/volume), the concentration of guanine used in the process of the present invention is about 30—100 microgram/ml.

20 The cultivation is carried out at a temperature between 20° and 40° C. and usually under aerobic conditions, for example by shaking the culture or by aerating and stirring a submerged culture. In the course of the cultivation the pH of the culture medium is kept between 5 and 8. When calcium carbonate is present in the culture medium to the extent of about 0.5 to 2.0% (weight/volume) a good accumulation of xanthosine can be obtained in the medium, one reason is thought to be the pH control action of calcium carbonate.

25 The progress of the fermentation may readily be followed by taking samples from the fermentation liquors at periodic intervals and measuring the ultraviolet absorption spectrum, or, more precisely, by chromatographing the sample on paper to separate the xanthosine and measuring the ultraviolet absorption spectrum to determine the amount of xanthosine contained in the culture medium. The maximum accumulation of xanthosine is normally reached after about 48 to 96 hours. Xanthylic acid, adenylic acid, and inosinic acid in addition to xanthosine may also be found in the culture medium depending on the conditions under which the

cultivation is carried out. After the cultivation is over, the culture broth is filtered and the filtrate is concentrated. Alcohol is added step-wise and the impurities which are precipitated are removed, alcohol is added to the filtrate which is cooled and allowed to stand to precipitate crystalline xanthosine. In another method, the filtrate of the culture medium is treated with active carbon to adsorb the xanthosine and the carbon is then treated with ammonia water and aqueous alcohol to extract the xanthosine. The extract is condensed, treated with alcohol, cooled, and the precipitate formed is isolated and dried to give xanthosine. The xanthosine recovered may, if necessary, be further recrystallised.

30 The following Examples, in which percentages are by weight per volume, illustrate the invention.

EXAMPLE 1.

Aerobacter aerogenes No. 5301 (ATCC No. 14304) was inoculated into a culture medium comprising 2% glucose, 1% peptone, 0.5% meat extract and 0.25% table salt and cultured at 28° C. for 24 hours to prepare the seed culture.

35 The fermentation medium was prepared as follows:— 50 g. of lactose, 10 g. of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g. of KH_2PO_4 , 0.5 g. of K_2HPO_4 , 0.25 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 74 mg. of guanine were dissolved in tap water to make the total volume of the solution 1 litre. The pH of the solution was adjusted to 7.4 and each 30 ml. portion thereof was poured into separate 250 ml. conical flasks. The flasks were sterilized and then sterilized CaCO_3 was added at the rate of 10 g./1 litre.

40 The seed culture was inoculated into the fermentation medium thus prepared, at the rate of 10% and cultured with shaking at 28° C. After 96 hours, the accumulated amount of xanthosine in the culture medium was 4.7 mg/ml. Beside xanthosine, small amounts of xanthylic acid, adenylic acid and inosinic acid were also found in the medium.

45 After termination of the cultivation, the culture medium was filtered to remove the cells and remaining calcium carbonate. The 450 ml. of filtrate thus obtained was concentrated under vacuum to 70 ml. and the precipitated calcium sulphate was removed, the filtrate was treated with 3 times its volume of alcohol, the precipitate removed and the filtrate was stood for several days in a cooled room. The precipitated xanthosine crystal thus obtained was filtered and dried to obtain 610 mg. of white needle-shaped crystals.

50 This crystalline material was identified as xanthosine by the combined results of elementary analysis, ultraviolet absorption spectrum analysis of its acidic, neutral and alkaline aqueous solutions, quantitative analysis of ribose and base, and paper chromatography.

EXAMPLE 2.

A fermentation medium was prepared as follows:— 50 g. of glucose, 10 g. of $(\text{NH}_4)_2\text{SO}_4$, 1 g. of K_2HPO_4 , 1 g. of KH_2PO_4 , 5 0.25 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 5 g. of yeast extract were dissolved in tap water to make a solution of total volume=1 litre. The pH of the solution was adjusted to 7.4 and sterilized CaCO_3 added at the rate of 10 g. 10 per litre of solution. Cultivation of *Aerobacter aerogenes* No. 5301 (ATCC No. 14304) was carried out in this fermentation medium using the same conditions as in Example 1.

15 After 72 hours cultivation, the xanthosine content of the culture medium reached 4.0 mg./ml.

WHAT WE CLAIM IS:—

1. A process for producing xanthosine 20 which comprises cultivating at 20° to 40° C. and a pH of 5 to 8 a guanine-requiring mutant strain of *Aerobacter aerogenes* in a nutrient medium comprising assimilable sources of carbon, nitrogen and inorganic salts 25 and guanine, guanosine or guanylic acid in an amount less than that required for the maximum growth of the microorganism and

separating xanthosine from the culture medium.

2. A process according to claim 1 wherein 30 the assimilable source of carbon is maltose, lactose, sucrose, glucose, fructose or mannitol.

3. A process according to claims 1 and 2 wherein the assimilable source of nitrogen is ammonium sulphate, chloride, nitrate, or 35 phosphate.

4. A process according to claims 1, 2 and 3 wherein the amount of guanine is 30—100 mg./litre and the concentration of assimilable carbon is 1 to 10 weight per volume percent. 40

5. A process according to any of the preceding claims wherein the mutant strain is *Aerobacter aerogenes* No. 5301 (ATCC No. 14304). 45

6. A process for producing xanthosine according to claim 1 substantially as herein-before described. 45

7. Xanthosine when obtained by the process claimed in any one of claims 1 to 6. 50

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