

PATENT SPECIFICATION

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(54) PRODUCTION OF CEPHALOSPORIN C

(71) We, TAKEDA YAKUHI KOGYO KABUSHIKI KAISHA, also known as TAKEDA CHEMICAL INDUSTRIES LTD., a body corporate organised according to the laws of Japan, of 27, Doshomachi 2-chome, Higashi-ku, Osaka, 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 process for the production of cephalosporin C.

Cephalosporin C (hereafter sometimes referred to briefly as CPC) is an antibiotic *per se* (H. W. Florey: Ann. Int. Med., 43, 480 (1955)) and also useful as a starting material for the synthesis of cephalosporin-group antibiotics (R. R. Chauvette et al. J. Am. Chem. Soc., 84, 3401 (1962)). It is known that cephalosporin C can be produced by growing certain microorganisms, for example, *Cephalosporium acremonium*, *Cephalosporium polyaleurum*, *Emericellopsis glabra*, and *Emericellopsis microspora*, cf. for example our Patent Specification No. 1,389,714. However, the methods utilising these microorganisms have the disadvantage that the CPC producing abilities of the strains are so low that the costs of production of CPC are of necessity considerable.

The present invention is based on the observation that among polyene-antibiotics-resistant CPC-producing microorganism belonging to the genus *Cephalosporium*, there was found a mutant, in a higher frequency than among other types of mutants, which is capable of accumulating CPC in a surprisingly high concentration, that is to say, 9,000 µg/ml. and even 10,000 µg/ml or more of CPC.

Accordingly, the present invention provides a process for the production of cephalosporin C, which comprises culturing a cephalosporin C-producing and polyene-antibiotics-resistant microorganism belonging to the genus *Cephalosporium* in a culture medium containing an assimilable carbon source and a digestible nitrogen source until cephalosporin

C has accumulated in the medium; and then recovering the cephalosporin C therefrom.

The microorganisms which are to be employed in this invention are microorganisms of the genus *Cephalosporium* which both produce CPC and are resistant to polyene antibiotics, and microorganisms belonging to the species *Cephalosporium acremonium* or *Cephalosporium polyaleurum* may be mentioned as preferred examples.

Polyene antibiotics are antibiotic substances possessing one or more multiple-membered lactone rings and having four to seven conjugated double bonds in their molecule, and include kabicidin (Japanese Patent Specification No. 247306), nystatin [Proc. Soc. Eper. Biol. and Med., 76, 93 (1961)], and trichomycin [Antibiotiki, 9, 291 (1964)], as typical examples. The term 'polyene antibiotics-resistant microorganism' is used herein to mean any microorganism which is induced from a parent CPC-producing microorganism of the genus *Cephalosporium* and has an increased resistance to polyene antibiotics of not less than 20 percent over the corresponding resistance of the parent, that is to say, a mutant for which the minimal inhibitory concentration (MIC) of a polyene antibiotic is not less than 120 percent of the minimal inhibitory concentration for the parent microorganisms using the agar dilution method under the following test conditions:

The basic medium for the test:

peptone 1%, meat extract 0.5%, NaCl 0.25%, agar 2.0% (pH 7.0).

Cultivation:

28°C, 4 days.

Induction of a polyene antibiotics-resistant microorganism can be performed by a method known *per se*, that is to say, in actual use in the art or described in the literature of the art. Thus, in one of the methods in use, the microorganism to which one wishes to impart resistance is grown on a fluid or agar medium containing the particular antibiotic in a concentration approximating its minimal inhibitory concentration and the concentra-

tion of the antibiotic in the medium is serially increased to adapt the microorganism until the desired resistant microorganism is obtained. In another method, the microbial cells are first treated with a conventional mutagen, e.g. ultraviolet-rays, X-rays or NTG(N - methyl - N' - nitro - N' - nitrosoguanidine) and, then, propagated on a fluid or agar medium containing the polyene antibiotic in a concentration in excess of its minimal inhibitory concentration and the resulting microbial growth is harvested as the resistant microorganism.

These methods may not only be practiced in repetition but also may be carried out in combination with other breeding methods of microorganisms. By such procedures, still improved results can be achieved.

The following are some typical examples of polyene antibiotics-resistant CPC-producing microorganisms belonging to the genus *Cephalosporium*: *Cephalosporium acremonium* K-121 (FERM-P No. 2285, ATCC-20427, IFO-9998) which has been induced from *C. acremonium* ATCC-14553 by UV treatment; *Cephalosporium acremonium* N-75 (FERM-P No. 2286, ATCC-20428, IFO-9997) which has also been induced from *C. acremonium* ATCC-14553 but by UV treatment; *Cephalosporium polyaleurum* K-771 which has been induced from *C. polyaleurum* 199 (ATCC-20359) by UV treatment; and *Cephalosporium polyaleurum* N-717 which is a mutant induced from *C. polyaleurum* Y-505 (ATCC-20360) by NTG treatment.

The numbers attached to the above microorganisms with the indications FERM-P, ATCC and IFO mean the deposition numbers at the Research Institute of Fermentation of the Agency of Industrial Science and Technology (FERM), Chiba, Japan; American Type Culture Collection (ATCC), Maryland, U.S.A.; and Institute for Fermentation, Osaka (IFO), Osaka, Japan, respectively.

Among the microorganisms mentioned above *Cephalosporium acremonium* K-121 and *Cephalosporium acremonium* N-75 are mutually similar and the most suitable microorganisms for the purpose of the present invention and their microbiological characteristics are as follows:

Colonies orbicular, dense, floccose, at first white, later very light rose-coloured; vegetative hyphae hyaline, sparsely septate, branched. Conidiophores arise as side branches on aerial hyphae, erect, simple, non-septate, 40-60 x 3 μ . Conidia numerous, elliptical or oblong, straight or curved, nearly hyaline, 4 x 1-1.5 μ .

The medium to be employed in the method of this invention may be one of the media used in the cultivation of conventional *Cephalosporium* strains.

Thus, as the assimilable carbon sources, there may be mentioned carbohydrates, e.g.

glucose, sucrose, starch, soluble starch, and waste molasses, *n* - paraffins, acetic acid, methanol, ethanol, glycerol, and sorbitol. As the digestible nitrogen sources, there may be mentioned, among others, inorganic nitrogen compounds, e.g. ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium phosphate, and potassium nitrate, and organic nitrogen compounds e.g. urea, meat extract, peptone, soybean meal, cotton seed cake, peanut cake, dried yeast, corn steep liquor, yeast extract, and casein. Metallic salts, for example, the sulphates, hydrochlorides, nitrates, carbonates, and phosphates, of sodium, potassium, calcium, magnesium, manganese, zinc, iron, and copper may be added, if necessary. Further, amino acids, e.g. methionine, cystine, cysteine, and serine, thiosulphates, fatty acid esters, and oils for example, lard oil, and olive oil, may also be incorporated, if necessary. As regards the conditions of cultivation, it is advantageous to grow the microorganisms under aerated submerged conditions. The temperature is preferably within the range of 15 to 37°C and, for still better results, within the range of 18 to 25°C. The pH may lie within the range of 2 to 10 and, preferably, within the range of 4 to 9, while the cultivation time may range from 48 to 480 hours and, preferably, from 72 to 360 hours.

Since most of the CPC is accumulated extracellularly in the broth, it is advantageous to centrifuge or filter the broth to remove the cells and to isolate CPC from the filtrate or supernatant. For example, the desired result can be achieved with advantage by using an ion exchange resin, activated carbon, non-ionic copolymer, and gel filtration, in a suitable combination.

Our Co-pending Application No. 42047/74 (Patent Specification No. 1,488,822) also relates to the production of cephalosporin C utilising certain microorganisms of the genus *Cephalosporium*. In that specification, however, the process described and claimed requires the use of a polyploid strain of *Cephalosporium*.

The following Examples illustrate the invention.

The quantitative determination of CPC was performed by an enzymatic assay method using the cephalosporinase of *Aerobacter cloacae* IFO 12937 (deposited at the Institute for Fermentation, Osaka). Thus, while CPC has an absorption maximum at 260 $m\mu$, this absorption is lost when cephalosporinase is permitted to act upon it. The determination of CPC was carried out by taking advantage of the difference.

In the following Examples the relationship between part(s) by weight and part(s) by volume is that between gram(s) and millilitre(s).

Example 1

A fermenter with 2,000 parts by volume in capacity is filled with 500 parts by volume of an inoculum medium made up of 3.0% of sucrose, 1.5% of meat extract, 0.5% of corn steep liquor and 0.15% of CaCO_3 and, after sterilization, inoculated with *Cephalosporium acremonium* K-121 (FERM-P No. 2285) (The MIC of kabicidin against this strain is 7.0 $\mu\text{g/ml}$, while the MIC of the same antibiotic against the parent strain is 2.5 $\mu\text{g/ml}$). The inoculated fermenter is incubated at 28°C for 3 days. Meanwhile a stainless-steel tank fermenter with 50,000 parts by volume in capacity is charged with 30,000 parts by volume of a medium made up of 6% of sucrose, 5% of glucose, 3% of peanut cake, 3% of soybean meal, 1.0% of DL-methionine and 0.15% of CaCO_3 . The medium is sterilized and cooled in the routine manner. This fermentation medium is aseptically inoculated with the inoculum culture prepared above and incubated at 28°C under sparging and agitation (aerated at 30,000 parts by volume/min and agitated at 250 r.p.m.). After a cultivation time of 190 hours, the fermentation broth is withdrawn and filtered to be free of solid matter.

The resultant filtrate (26,000 parts by volume) is assayed for CPC content. The titer found of CPC is 10,100 $\mu\text{g/ml}$.

The CPC is fractionally recovered in the following manner. The above filtrate (26,000 parts by volume) is passed down through a column of activated carbon (20,000 parts by volume) whereby the CPC is adsorbed on the carbon. The column is first washed well with water and, then CPC is eluted with a 5% aqueous solution of butanol containing 0.01 N NaOH, whereupon 33,000 parts by volume of CPC fractions are obtained. These CPC fractions are pooled and concentrated.

The concentrate is neutralized to pH 7.0 with NaOH and a sufficient amount of ethanol is added to the concentrate to give an ethanol concentration of 70% (V/V). The procedure gives 182 parts by weight of crude crystals of CPC sodium dihydrate.

Example 2

A fermenter with 2,000 parts by volume in capacity is filled with 500 parts by volume of an inoculum medium made up of 3.0% of sucrose, 1.5% of meat extract, 0.5% of corn steep liquor and 0.15% of CaCO_3 and, after sterilization, inoculated with *Cephalosporium acremonium* N-75 (FERM-P No. 2286) (The MIC of nystatin against this strain is 6.0 $\mu\text{g/ml}$ while the MIC of the same antibiotic against the parent is 1.7 $\mu\text{g/ml}$). The inoculated flask is incubated at 28°C for 3 days.

Meanwhile, a stainless-steel fermenter with 50,000 parts by volume in capacity is charged with 30,000 parts by volume of a fermenta-

tion medium made up of 6% of sucrose, 5% of glucose, 3.0% of peanut cake, 3% of soybean meal, 1.0% of DL-methionine and 0.15% of CaCO_3 , and the medium is sterilized and cooled in the routine manner. This fermentation medium is aseptically inoculated with the inoculum culture prepared above and incubated at 28°C under sparging and agitation (aerated at 30,000 parts by volume/min., agitated at 250 r.p.m.). After 190 hour cultivation time, the fermentation broth is withdrawn and filtered to remove the solid matter. The resultant filtrate (26,000 parts by volume) is assayed for CPC. The titer found of CPC is 9,500 $\mu\text{g/ml}$. Thereafter, the procedure of Example 1 is followed to obtain 165 parts by weight of CPC sodium dihydrate.

WHAT WE CLAIM IS:—

1. A process for the production of cephalosporin C, which comprises culturing a cephalosporin C-producing and polyene-antibiotics-resistant microorganism belonging to the genus *Cephalosporium* in a culture medium containing an assimilable carbon source and a digestible nitrogen source until cephalosporin C has accumulated in the medium; and then recovering the cephalosporin C therefrom.
2. A process according to claim 1, wherein the microorganism is a strain of *Cephalosporium acremonium*.
3. A process according to claim 1, wherein the microorganism is a strain of *Cephalosporium polyaleurum*.
4. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* K-121 (FERM-P No. 2285, IFO-9998, ATCC-20427).
5. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* N-75 (FERM-P. No. 2286, IFO-9997, ATCC-20428).
6. A process according to any one of claims 1 to 5, wherein the microorganism is cultured at a temperature within the range of from 15° to 37°C.
7. A process according to claim 6, wherein the temperature is from 18° to 35°C.
8. A process according to any one of claims 1 to 7, wherein the pH of the culture medium is within the range of from 2 to 10.
9. A process according to claim 8, wherein the pH is from 4 to 9.
10. A process according to claim 1, conducted substantially as described in Example 1 or Example 2 herein.
11. Cephalosporin C whenever produced by a process according to any one of claims 1 to 10.
12. A cephalosporin C-producing and polyene-antibiotics-resistant microorganism of the genus *Cephalosporium* which has been induced by a method suitable for the production of microorganisms resistant to an antibiotic,



(11)

Example 1

A fermenter with 2,000 parts by volume in capacity is filled with 500 parts by volume of an inoculum medium made up of 3.0% of sucrose, 1.5% of meat extract, 0.5% of corn steep liquor and 0.15% of CaCO_3 and, after sterilization, inoculated with *Cephalosporium acremonium* K-121 (FERM-P No. 2285) (The MIC of kabicidin against this strain is 7.0 $\mu\text{g/ml}$, while the MIC of the same antibiotic against the parent strain is 2.5 $\mu\text{g/ml}$). The inoculated fermenter is incubated at 28°C for 3 days. Meanwhile a stainless-steel tank fermenter with 50,000 parts by volume in capacity is charged with 30,000 parts by volume of a medium made up of 6% of sucrose, 5% of glucose, 3% of peanut cake, 3% of soybean meal, 1.0% of DL-methionine and 0.15% of CaCO_3 . The medium is sterilized and cooled in the routine manner. This fermentation medium is aseptically inoculated with the inoculum culture prepared above and incubated at 28°C under sparging and agitation (aerated at 30,000 parts by volume/min., agitated at 250 r.p.m.). After 190 hours, the fermentation broth is withdrawn and filtered to be free of solid matter.

The resultant filtrate (26,000 parts by volume) is assayed for CPC content. The titer found of CPC is 10,100 $\mu\text{g/ml}$.

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The concentrate is neutralized to pH 7.0 with NaOH and a sufficient amount of ethanol is added to the concentrate to give an ethanol concentration of 70% (V/V). The procedure gives 182 parts by weight of crude crystals of CPC sodium dihydrate.

Example 2

A fermenter with 2,000 parts by volume in capacity is filled with 500 parts by volume of an inoculum medium made up of 3.0% of sucrose, 1.5% of meat extract, 0.5% of corn steep liquor and 0.15% of CaCO_3 and, after sterilization, inoculated with *Cephalosporium acremonium* N-75 (FERM-P No. 2286) (The MIC of nystatin against this strain is 6.0 $\mu\text{g/ml}$ while the MIC of the same antibiotic against the parent is 1.7 $\mu\text{g/ml}$). The inoculated flask is incubated at 28°C for 3 days.

Meanwhile, a stainless-steel fermenter with 50,000 parts by volume in capacity is charged with 30,000 parts by volume of a fermenta-

tion medium made up of 6% of sucrose, 5% of glucose, 3.0% of peanut cake, 3% of soybean meal, 1.0% of DL-methionine and 0.15% of CaCO_3 , and the medium is sterilized and cooled in the routine manner. This fermentation medium is aseptically inoculated with the inoculum culture prepared above and incubated at 28°C under sparging and agitation (aerated at 30,000 parts by volume/min., agitated at 250 r.p.m.). After 190 hour cultivation time, the fermentation broth is withdrawn and filtered to remove the solid matter. The resultant filtrate (26,000 parts by volume) is assayed for CPC. The titer found of CPC is 9,500 $\mu\text{g/ml}$. Thereafter, the procedure of Example 1 is followed to obtain 165 parts by weight of CPC sodium dihydrate.

WHAT WE CLAIM IS:—

1. A process for the production of cephalosporin C, which comprises culturing a cephalosporin C-producing and polyene-antibiotics-resistant microorganism belonging to the genus *Cephalosporium* in a culture medium containing an assimilable carbon source and a digestible nitrogen source until cephalosporin C has accumulated in the medium; and then recovering the cephalosporin C therefrom.

2. A process according to claim 1, wherein the microorganism is a strain of *Cephalosporium acremonium*.

3. A process according to claim 1, wherein the microorganism is a strain of *Cephalosporium polyaleurum*.

4. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* K-121 (FERM-P No. 2285, IFO-9998, ATCC-20427).

5. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* N-75 (FERM-P. No. 2286, IFO-9997, ATCC-20428).

6. A process according to any one of claims 1 to 5, wherein the microorganism is cultured at a temperature within the range of from 15° to 37°C.

7. A process according to claim 6, wherein the temperature is from 18° to 35°C.

8. A process according to any one of claims 1 to 7, wherein the pH of the culture medium is within the range of from 2 to 10.

9. A process according to claim 8, wherein the pH is from 4 to 9.

10. A process according to claim 1, conducted substantially as described in Example 1 or Example 2 herein.

11. Cephalosporin C whenever produced by a process according to any one of claims 1 to 10.

12. A cephalosporin C-producing and polyene-antibiotics-resistant microorganism of the genus *Cephalosporium* which has been induced by a method suitable for the production of microorganisms resistant to an antibiotic,

(12)



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from a cephalosporin C-producing micro-
organism of the genus *Cephalosporium*.

sporium acremonium ATCC-14553 or a
mutant thereof.

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13. A microorganism as claimed in claim
12, which has been induced from a cephalo-
sporin C-producing strain of *Cephalosporium*
5 *acremonium* or *Cephalosporium polyaleurum*.

14. A microorganism as claimed in claim
13, which has been induced from *Cephalo-*

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