

PATENT SPECIFICATION

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(54) PRODUCTION OF CEPHALOSPORIN C

(71) We, TAKEDA YAKUHIN 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 the production of cephalosporin-C (hereinafter abbreviated to "CPC") by fermentation processes.

Methods by which increased yields of CPC produced by fermentation processes may be obtained have been investigated, cf, for example, our Patent Specification No. 1,389,714, but few methods are known in which selective breeding of microbial strains is involved. The only methods known at the present time are those which utilise a strain whose metabolism and biosynthesis of organosulphur compounds have been blocked (Japanese Patent Application Laid Open No. 1828/1971) and those in which the CPC-producing ability of microorganisms has been somehow enhanced (USP No. 3,082,155 and Japanese Patent Application Laid Open No. 26986/1973). Moreover, in regard to the abilities of microorganisms to produce CPC, the fermentation yields are 760 µg/ml at best in the case of the Japanese Patent Application Laid Open No. 1828/1971, about 500 µg/ml in the case of USP No. 3,082,155 and, even in the most satisfactory of all the cases heretofore reported, Japanese Patent Application Laid Open No. 26986/1973, the attainable potency is not more than 5,000 µg/ml.

The present invention is based on the observation that microorganisms possessing excellent CPC-producing abilities can be obtained at a higher frequency from among polyploid microorganisms induced by polyploidizing treatment of CPC-producing microorganisms belonging to the genus *Cephalosporium* than from among other mutants induced by other conventional mutation treatment, and that some

of the polyploid microorganisms are able to accumulate CPC extracellularly in unprecedentedly high concentrations.

Although attempts have heretofore been made to improve the fermentation yields of desired products by the use of microbial polyploid strains, none of reported cases has been industrially successful, either because of the failure to achieve satisfactory results or because of the difficulty with which these strains lend themselves to genetic fixation of characters. In particular, there is not a single reported case of any such attempt in connection with CPC fermentation.

The present invention provides a process for the production of CPC which comprises cultivating a polyploid cephalosporin C-producing microorganism induced from a parent microorganism of the genus *Cephalosporium* in a culture medium comprising an assimilable carbon source and a digestible nitrogen source until cephalosporin C has accumulated extracellularly in the medium, and recovering the cephalosporin C therefrom.

As the parent strains of microorganisms that are employed in this invention, use can be made of any suitable CPC producing strain of the genus *Cephalosporium*, for example, *Cephalosporium acremonium* or *Cephalosporium polyaleurum*.

As typical strains of the species, there may be mentioned, among others, *Cephalosporium polyaleurum* Y-505 (FERM—P No. 1160), *Cephalosporium acremonium* ATCC—11550, and *Cephalosporium acremonium* ATCC—14553, and various mutants induced from these strains.

The FERM—P and ATCC numbers indicate the accession numbers of the organisms at the Research Institute of Fermentation of the Agency of Industrial Science and Technology, Chiba, Japan and at American Type Culture Collection, Rockville, Maryland, U.S.A., respectively.

The methods by which polyploid microorganisms are produced are known *per se*, that is to say they are in actual use in the art or described in the literature of the art.

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The following two procedures may be cited as principal procedures for inducing desired polyploid microorganisms from parent microorganisms.

- 5 One of them is the procedure whereby microbial cells are polyploidized by means of a chemical agent. As examples of suitable chemical agents, there may be mentioned camphor, acenaphthene, and colchicine. While
- 10 the conditions of treatment vary with the particular microorganism, type of chemical agent and other factors, the following procedure generally can be followed with success: In the case of camphor or acenaphthene, for instance,
- 15 microbial cells are exposed to a vapour of the chemical for a period of from a few to tens of hours, after which they are removed from the ambient vapour and further cultivated. In a variant of this method, the cells are cultivated from the start on an agar plate contain-
- 20 ing the chemical. The grown cells taken from the resulting colonies are examined, e.g. under a microscope, and those colonies made up of giant cells are selected.
- 25 By either of the above-described procedures there may be obtained the desired polyploid microorganism. For example, a polyploid microorganism can be obtained by the method described in Journal of General and Applied
- 30 Microbiology 2, 345 (1956).

These procedures can not only be followed in repetition but can be followed in combination with other methods of breeding and selecting some specific microbial strains and, in such instances, improved results are some-

times obtained. The polyploid strains thus obtained can be easily isolated, if desired, by a conventional pure-isolation method, for example by micro-manipulation.

To illustrate, *Cephalosporium acremonium* ATCC—14553 as a parent strain was treated with camphor and the CPC-producing abilities of the resulting polyploid strains were investigated. The investigation resulted in the finding of a number of strains which can accumulate CPC in the medium in the increased yields for example, more than 7,000 $\mu\text{g/ml}$, with *Cephalosporium acremonium* 2M—16 (FERM—P No. 2283, ATCC—2045, IFO—9999), in particular, giving an unprecedentedly high yield of CPC. The taxonomic characters of this strain were found to be essentially identical with those of *Cephalosporium* sp. ATCC—14553 (Japanese Patent Publication No. 4437/1965) except for the differences noted in Table 1.

The IFO number indicates the accession numbers of the organisms at the Institute for Fermentation, Osaka, Japan.

TABLE 1

Strain	Size of arthrospore		Number of nuclei per arthrospore (A)	DNA content per arthrospore (B) (μg)	Amount of DNA per nucleus (B/A) (μg)
	Major diameter (μ)	Minor diameter (μ)			
<i>Cephalosporium acremonium</i> ATCC-14553	7.2	3.6	1.0	0.30×10^{-7}	0.30×10^{-7}
<i>Cephalosporium acremonium</i> 2M-16	11.2	4.7	3.2	1.98×10^{-7}	0.62×10^{-7}

(Note: All the values given in the above table are the results of analysis of the arthrospores obtained after 2 weeks' incubation at 28°C on an agar medium composed of 0.25% yeast extract, 0.5% malt extract, 0.1% casein hydrolyzate (Casamino acid), 2.0% sucrose, 1.5% glycerol and 2.0% agar. The dimensions of the arthrospore and the number of nuclei are mean of results for approximately 100 arthrospores. The mean dimensions of conidia are $3.5 \mu \times 7.1 \mu$ in the case of ATCC-14553 and $4.3 \mu \times 8.6 \mu$ in the case of 2M-16.)

The second genetic method typically comprises the procedure described in Journal of General and Applied Microbiology 2, 401 (1956). Thus, the two strains to be crossed are differently marked beforehand, e.g. with different amino acid or vitamin requirements. They are crossed and grown on a minimal

medium (e.g. a medium made up of 3.0% sucrose, 0.3% NH_4NO_3 , 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, a trace of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.5% agar) in the conventional manner. The conidia of grown colonies are collected and plated onto a minimal medium such as the one indicated above and the colonies formed are investigated. Most of the growth consists of polyploid strains.

If necessary, these methods can not only be practiced in repetition but also be carried out in combination with other selective breeding procedures, and improved results can be obtained thereby. The resultant polyploid strain can be easily isolated, if desired, by a known pure-isolation method such as micro-manipulation.

When the second genetic method of ploidy described above was carried out crossing *Cephalosporium acremonium* LM-5,

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5 which is a methionine - leucine - requiring mutant, and *Cephalosporium acremonium* AP-78, an arginine-phenylalanine-requiring mutant, both induced from *Cephalosporium acremonium* ATCC-14553 as the parent strain, a number of polyploid strains were obtained.

10 An investigation of the abilities of these polyploid strains to accumulate CPC resulted in the finding of many superior CPC-producing strains which can accumulate CPC extracellu-

larly in the concentration of, for example, more than 7,000 mg/ml, with *Cephalosporium acremonium* LA-101 (FERM-P No. 2284, ATCC-20426, IFO-30001), in particular, giving an unprecedentedly high yield of CPC.

The taxonomic characters of this strain were found to be essentially identical with the characters of *Cephalosporium* sp. ATCC-14553 except for the differences noted in Table 2.

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TABLE 2

Strain	Size of conidium		Number of nuclei per conidium (A)	DNA content per conidium (B) (μg)	DNA content per nucleus (B/A) (μg)
	Major diameter (μ)	Minor diameter (μ)			
<i>Cephalosporium acremonium</i> ATCC-14553	10.5	2.5	1	0.691×10^{-7}	0.691×10^{-7}
<i>Cephalosporium acremonium</i> LM-5	7.5	2.3	1	0.670×10^{-7}	0.670×10^{-7}
<i>Cephalosporium acremonium</i> AP-78	7.3	2.7	1	0.710×10^{-7}	0.710×10^{-7}
<i>Cephalosporium acremonium</i> LA-101	7.9	3.3	1	1.380×10^{-7}	1.380×10^{-7}

(Note: Each of the values given above is the result of analysis of the conidia harvested from a slant culture grown by the procedure set forth in the footnote to Table 1. The dimensions of conidia and the number of nuclei are the mean values for about 100 spores.)

To cultivate these polyploid strains, the known methods for culture of other CPC producers of the genus *Cephalosporium* or those of the genus *Emicelopsis* can be employed to advantage.

As the carbon sources that can be employed, there may be mentioned, for example, glucose, sucrose, starch, soluble starch, waste molasses, *n*-paraffins, acetic acid, methanol, and ethanol. As organic nitrogen sources, mention may be made of urea, meat extract, peptone, soybean meal, cotton seed cake, peanut cake, dried yeast, and corn steep liquor, while ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium phosphate, and potassium nitrate may be mentioned as useful inorganic

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sources of nitrogen. If necessary, there may be added such metal salts as the SO_4 , Cl , NO_3 , CO_3 , PO_4 , and other salts of Na, K, Ca, Mg, Mn, Zn, Fe, and Cu.

- 5 Furthermore, there may also be added, as required, amino acids, e.g. methionine, cysteine, and serine, thiosulphates, fatty acid esters, oils, e.g. lard oil and olive oil, and other agents which assist in the production of CPC, whereby the concentration of accumulated CPC can be further increased.

- 10 As to the conditions of cultivation, aerated submerged culture is generally advantageous. The temperature is desirably within the range of from 18 to 35°C. Satisfactory results are obtained when the pH is maintained within the range of about 2 to 10 and, preferably, between 4 and 9. A cultivation time of 3 to 15 days is generally sufficient. Most of the CPC elaborated is found extracellularly in the culture broth and, therefore, to recover the antibiotic, it is advantageous to remove the cells from the broth by centrifugation or filtration and, then, to isolate CPC from the supernatant or filtrate. To isolate the CPC, those procedures generally used for the fractional recovery of CPC can be followed with success. For example, the desired result can be obtained with advantage by utilizing an ion exchange resin, activated carbon, nonionic copolymer resin, and gel filtration medium, in a suitable combination.

- 20 Our Co-pending Application No. 42042/74 (Patent Specification No. 1,488,821) also relates to the production of cephalosporin C utilizing certain microorganisms of the genus *Cephalosporium*. In that specification, however, the process described and claimed requires the use of polyene-antibiotics resistant strain of *Cephalosporium*.

- 40 The following Examples illustrate the invention.

Example 1.

- 45 A Sakaguchi flask of 2-litre capacity is filled with 500 ml 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* 2M-16.
- 50 The flask is incubated on a reciprocating shaker at 28°C for 3 days. Meanwhile, a 50-litre fermentation tank of stainless steel is charged with 30 litres 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 , and the medium is sterilized and cooled in the routine manner. The medium is then aseptically inoculated with the above seed culture and incubated at 28°C under sparging and agitation (30 l. per min. aeration, 250 r.p.m.).

- 60 After 190 hours of cultivation, the culture broth is withdrawn and filtered free of the solids. The resultant filtrate (25 l.) is assayed

for CPC. The potency found is 8,200 $\mu\text{g/ml}$. The fractional isolation of CPC is carried out in the following manner: The filtrate (25 l.) is run down a column of activated carbon (20 l.), whereby the CPC is adsorbed on the carbon. The carbon is first washed well with water and, then, eluted with 5% aqueous butanol containing 0.01 N NaOH, whereupon 31 l. of CPC fractions are obtained. These fractions are pooled, concentrated and neutralized to pH 7.0 with NaOH. Finally, ethanol is added to the concentrate to a concentration of 50% (V/V).

The procedure gives 143 g. crude crystals of CPC sodium dihydrate.

The potency of CPC is measured by an enzymatic assay method using the cephalosporinase of *Aerobacter cloacae* (Biochemical Journal, 116, 385 (1970)).

Example 2.

The same cultivation as Example 1 is carried out, except that the strain used is *Cephalosporium acremonium* LA-101. The potency of CPC in filtered broth is 7,900 $\mu\text{g/ml}$.

WHAT WE CLAIM IS:—

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

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

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

4. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* 2M-16 (ATCC-20425).

5. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* LA-101 (ATCC-20426).

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 18° to 35°C.

7. A process according to any one of claims 1 to 6, wherein the pH of the culture medium is within the range of from 2 to 10.

8. A process according to claim 7, wherein the pH is from 4 to 9.

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

10. Cephalosporin C whenever produced by a process according to any one of claims 1 to 9.

11. A cephalosporin C-producing polyploid

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sources of nitrogen. If necessary, there may be added such metal salts as the SO_4 , Cl , NO_3 , CO_3 , PO_4 , and other salts of Na, K, Ca, Mg, Mn, Zn, Fe, and Cu.

5 Furthermore, there may also be added, as required, amino acids, e.g. methionine, cysteine, and serine, thiosulphates, fatty acid esters, oils, e.g. lard oil and olive oil, and other agents which assist in the production of CPC, whereby the concentration of accumulated CPC can be further increased.

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

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

4. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* 2M-16 (ATCC-20425).

5. A process according to claim 2, wherein the microorganism is *Cephalosporium acremonium* LA-101 (ATCC-20426).

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 18° to 35°C.

7. A process according to any one of claims 1 to 6, wherein the pH of the culture medium is within the range of from 2 to 10.

8. A process according to claim 7, wherein the pH is from 4 to 9.

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

10. Cephalosporin C whenever produced by a process according to any one of claims 1 to 9.

11. A cephalosporin C-producing polyploid



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- microorganism belonging to the genus *Cephalosporium* which has been induced by a method suitable for the production of polyploid microorganisms from a cephalosporin C-producing microorganism of the genus *Cephalosporium*. (FERM—P No. 1160), *Cephalosporium acremonium* ATCC—11550 or *Cephalosporium acremonium* ATCC—14553, or a mutant thereof. 15
- 5 12. A polyploid microorganism as claimed in claim 11, which has been induced from a cephalosporin C-producing strain of *Cephalosporium acremonium* or *Cephalosporium polyaleurum*. 14. *Cephalosporium acremonium* 2M—16 (ATCC—20425). 20
- 10 13. A polyploid microorganism as claimed in claim 12, which has been induced from *Cephalosporium polyaleurum* Y—505 15. *Cephalosporium acremonium* LA—101 (ATCC—20426).

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