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(54) METHOD FOR PRODUCING CITRIC ACID BY FERMENTATION

(71) We, TAKEDA YAKUHIN KOGYO KABUSHIKI KAISHA, (TAKEDA CHEMICAL INDUSTRIES, LTD.), of 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, a corporate body organised under the laws of 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 citric acid. More particularly, this invention relates to a method for producing citric acid, which comprises inoculating a citric acid-accumulating and hydrocarbon-assimilating strain of a yeast belonging to the genus *Candida* in an aqueous culture medium containing, as the main carbon source at least one of normal paraffins with from 9 to 20 carbon atoms, incubating the culture at a specific pH; and recovering the so-accumulated citric acid therefrom.

Citric acid is in great demand and used for example, as an acidulant in beverages and in pharmaceutical syrups. The present inventors found that yeasts have the ability of accumulating citric acid in a medium containing such carbon sources as glucose and sucrose, and completed a method for the production of citric acid. However, this method requires relatively expensive carbohydrates as the carbon sources and generally accompanies the production of polyhydric alcohols, resulting in a low yield.

In an attempt to overcome these disadvantages, the present inventors have further found that there exist citric acid-accumulating and hydrocarbon-assimilating strains of yeasts, that most of the yeasts belong to the genus *Candida*, that a suitable pH control of the culture medium during the incubation enables the strains to accumulate citric acid abundantly, and that the yield is high and the recovery is easily conducted.

We have sought to provide a method for producing citric acid from less expensive carbon sources in a good yield.

The invention provides a method of producing citric acid by incubating a yeast which is capable of utilizing hydrocarbons, as a sole carbon source, particularly normal paraffins with from 9 to 20 carbon atoms and which is also capable of accumulating citric acid in a medium containing one or more of the said hydrocarbons.

In the method of the present invention the yeast employed must be capable of utilizing the hydrocarbons mentioned above and of accumulating citric acid. We have found that strains of yeast belonging to the genus *Candida* fulfil these conditions, e.g. *Candida lipolytica*, *Candida tropicalis*, *Candida intermedia*, *Candida parapsilosis* and *Candida guilliermondii*.

The microbial characteristics of yeasts are not generally fixed and this applies to the characteristics of these belonging to the genus *Candida*. Otherwise stated, there are many mutants and variants, and among the mutants and variants, regardless of whether the variation is caused naturally or artificially, for example, the X-ray, ultraviolet ray, or by the action of chemical reagents, any one can be employed in the method of the present invention so long as it is hydrocarbon-assimilating and citric acid-accumulating.

The culture medium to be employed in this invention is required to contain at least one of the normal paraffins with from 9 to 20 carbon atoms, for example, nonane, decane, dodecane, tetradecane, hexadecane, octadecane and eicosane. There may also be exemplified such impure materials as kerosene, heavy paraffins, heavy gas oil and gas oil each containing at least about 10% (volume/volume) or normal paraffins with from 9 to 20 carbon atoms. The hydrocarbons are generally used in such an amount as to make the concentration in the culture medium of the normal paraffins with from 9 to 20 carbon atoms as a whole about 3 to 20% (volume/volume) in the culture medium.

As these hydrocarbons are scarcely soluble in water, the addition thereof to an aqueous culture medium is practically carried out with

stirring or shaking to prepare a suspension containing very fine particles. If desired, a suspending agent, e.g. a surfactant of the type of polyoxyethylene sorbitan monostearate may be employed.

These hydrocarbons are by themselves sufficient carbon sources, but, if desired, commonly employable carbon sources such as carbohydrate (e.g. glucose) may be used together with the hydrocarbons.

As digestable nitrogen sources, there may be exemplified such inorganic compounds as ammonium chloride, ammonium sulfate, and ammonium phosphate, such organic nitrogen compounds as ammonium acetate, peptone, urea, yeast extract and corn steep liquor. In the case of organic nitrogen compounds or materials being employed, they may be consumed not only as nitrogen sources but also as a part of carbon sources.

Further a small quantity of inorganic salts such as sodium chloride, phosphates, salts of metals such as calcium, zinc, iron and magnesium may be added to the medium, and if necessary, conventional nutrient factors or antifoaming agents such as vitamins animal oil, vegetable oil or mineral oil may be added.

In carrying out the process of this invention, it is preferable to employ a liquid culture medium, and the incubation is carried out aerobically, i.e. with aeration under static or submerged conditions.

Incubation conditions such as the pH of the medium and the incubation temperature should be controlled so that the objective citric acid may be accumulated in the maximum amount. The pH of the culture is maintained at from 4 to 7.5, more advantageously from 5 to 6.5.

As the pH goes down in the course of incubation, the addition of salts of an alkali metal, an alkaline earth metal or ammonia as well as bases as pH-adjusting agents is recommendable in order to keep the pH in an optimal range. The salts or bases include, for example, calcium carbonate, calcium acetate, sodium hydroxide, calcium hydroxide and aqueous ammonia. Addition of them is effected before or with the start of cultivation or at a suitable stage in the course of cultivation, and in the former case, salts are more preferably employed.

Generally, calcium ion as well as magnesium ion promotes the accumulation of citric acid. The incubation is carried out at a temperature of from 15° to 40°C, preferably at a temperature of from 20° to 30°C.

The accumulated citric acid in the culture broth is recovered therefrom by applying per se conventional means such as filtration, centrifugation, heating, column chromatography, activated carbon treatment, concentration or sedimentation, solely or in combination. If necessary, the pH of the culture broth

may be adjusted. By way of illustration, when there exists insoluble calcium citrate in the culture broth, it is preferable to acidify the broth with hydrochloric acid to solubilize the said salt, followed by further separation procedures.

If desired, after being separated from yeast cells, the culture liquid is neutralized with sodium hydroxide, calcium carbonate or milk of lime. The solution thus obtained is kept standing at room temperature or under heating to precipitate calcium nitrate, followed by filtration or centrifugation and drying to give calcium nitrate as a white powder. The powder is, if needed, further purified by the conventional means such as neutralization, filtration, concentration or recrystallization.

Preferred embodiments of the invention are shown in the following examples, but they are not to be construed as limitative of the present invention. Percentages are calculated on the weight per volume basis, and yields are calculated on weight of the produced citric acid per weight of consumed normal paraffins. The relationship between part(s) by weight and part(s) by volume is the same as that between gram(s) and millilitre(s).

The IFO numbers put after the name of yeasts employed in the examples are the respective accession numbers at Institute for Fermentation, Osaka, Japan.

EXAMPLE 1.

A culture of *Candida lipolytica* IFO-1437 is inoculated into 250 parts by volume of a sterilized aqueous medium with a pH of 7.2, composed of ammonia chloride (0.4%), potassium dihydrogen phosphate (0.05%), magnesium sulfate (0.05% as heptahydrate), yeast extract (0.1%), calcium carbonate (3%) plus 10 parts by weight of sterilized n-decane, and is incubated with agitation and aeration at 26°C for 7 days.

200 Parts by volume of the culture broth are adjusted to pH 2 with hydrochloric acid and centrifuged to separate yeast cells. The culture liquid is neutralized by sodium hydroxide with heating to give precipitates. After cooling, the precipitates are filtered to give 5.8 parts by weight of calcium citrate (corresponding to 4.4 parts by weight of anhydrous citric acid). A 50% solution of sulfuric acid is added dropwise to the thus obtained calcium nitrate suspended in ten times as much water with stirring until the existence of excess sulfate is confirmed by the addition of barium chloride.

The whole is heated to boiling for about 30 minutes and concentrated to a syrup at a temperature between about 50° to 60°C under a reduced pressure while the precipitated calcium is filtered off, if present. The concentrate is kept in an ice room to yield 5.96 parts by weight of anhydrous citric acid. (Yield 107% as anhydrate)

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EXAMPLE 2.

A culture of *Candida lipolytica* IFO-1437 is inoculated into 250 parts by volume of a sterilized aqueous medium with a pH of 5.0 composed of ammonium chloride (0.4%), potassium dihydrogen phosphate (0.05%), magnesium sulfate heptahydrate (0.05%), yeast extract (0.1%), n-hexadecane (4%), calcium chloride dihydrate (0.01%) plus 0.0025 part by weight of bromocresol green, and is incubated with agitation and aeration at 28°C for 8 days. During the incubation, the pH of the culture medium is adjusted with a 5% solution of calcium hydroxide so as to keep the color of the medium green. 200 Parts by volume of the thus-obtained culture broth is subjected to further recovering procedures as in Example 1 to give 6.4 parts by weight of citric acid. Yield 114%)

EXAMPLE 3.

A culture of *Candida tropicalis* IFO-0589 is inoculated into 250 parts by volume of a sterilized aqueous medium composed of ammonium sulfate (0.4%), potassium dihydrogen phosphate (0.1%) magnesium sulfate heptahydrate (0.1%) ferric sulfate heptahydrate (0.001%), calcium chloride (0.01%), manganese sulfate tetrahydrate (0.001%), yeast extract (0.05%), n-tetradecane (5%) (pH 5.0), and incubated with agitation and aeration at 28°C for 8 days while the pH of the culture broth is kept at 5.0 with the addition of sodium hydroxide, at the end of the incubation period 16.7 mg. of citric acid per ml. of the broth are accumulated.

EXAMPLE 4.

A culture of *Candida intermedia* IFO-0761 is inoculated into 250 parts by volume of a sterilized aqueous medium with a pH of 6.8 composed of ammonium sulfate (0.4%), potassium dihydrogen phosphate (0.05%), magnesium sulfate heptahydrate (0.05%), yeast extract (0.1%) calcium carbonate (3%) and distilled components including 82% n-paraffin with from 12 to 16 carbon atoms (5%), and incubated under agitation and aeration at 30°C for 6 days, at the end of the incubation period, 12.4 mg. of citric acid per ml. of the culture broth are accumulated.

EXAMPLE 5.

A culture of *Candida parapsilosis* IFO-0708 is inoculated into 250 parts by volume of a sterilized medium with the pH of 7.2 composed of the same components as in Example 1 plus calcium acetate (1%) and 10 parts by weight of distilled fraction including n-paraffin with from 16 to 20 carbon atoms,

and is incubated under agitation and aeration for 8 days, at the end of the incubation period, 18.1 mg. of citric acid per ml. of culture broth are accumulated. 60

EXAMPLE 6.

In the same manner as in Example 2, employing a medium as described in Example 2, 18.4 mg. of citric acid per ml. of the culture broth are accumulated by the incubation of *Candida guilliermondii* IFO-0838. 65

WHAT WE CLAIM IS:—

1. A method of producing citric acid which comprises inoculating a citric acid-accumulating and hydrocarbon-assimilating strain of a yeast belonging to the genus *Candida* in an aqueous culture medium containing at least one of the normal paraffins with from 9 to 20 carbon atoms as the main carbon source; incubating the culture at a pH of from 4 to 7.5 until citric acid is accumulated in the culture broth; and recovering the so-accumulated citric acid therefrom. 70

2. A method according to claim 1, wherein the incubation temperature is from 15° to 40°C. 75

3. A method according to claim 1 or 2, wherein the pH of the culture medium is kept from 5 to 6.5. 80

4. A method according to any of claims 1 to 3, wherein the normal paraffins are used in an amount of from 3 to 20% by volume of the culture medium. 85

5. A method according to any of claims 1 to 4, wherein the yeast is *Candida Lipolytica*. 90

6. A method according to any of claims 1 to 4, wherein the yeast is *Candida tropicalis*. 95

7. A method according to any of claims 1 to 4, wherein the yeast is *Candida intermedia*. 100

8. A method according to any of claims 1 to 4, wherein the yeast is *Candida parapsilosis*. 105

9. A method according to any of claims 1 to 4, wherein the yeast is *Candida guilliermondii*. 110

10. A method according to claim 5, wherein the yeast is *Candida lipolytica* IFO-1437. 115

11. A method according to claim 6, wherein the yeast is *Candida tropicalis* IFO-0589. 120

12. A method according to claim 7, wherein the yeast is *Candida intermedia* IFO-0761. 125

13. A method according to claim 8, wherein the yeast is *Candida parapsilosis* IFO-0708. 130

14. A method according to claim 9, wherein the yeast is *Candida guilliermondii* IFO-0838. 135



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EXAMPLE 2.

5 A culture of *Candida lipolytica* IFO-1437 is inoculated into 250 parts by volume of a sterilized aqueous medium with a pH of 5.0 composed of ammonium chloride (0.4%), potassium dihydrogen phosphate (0.05%), magnesium sulfate heptahydrate (0.05%), yeast extract (0.1%), n-hexadecane (4%), calcium chloride dihydrate (0.01%) plus 0.0025 part by weight of bromocresol green, and is incubated with agitation and aeration at 28°C for 8 days. During the incubation, the pH of the culture medium is adjusted with a 5% solution of calcium hydroxide so as to keep the color of the medium green. 200 Parts by volume of the thus-obtained culture broth is subjected to further recovering procedures as in Example 1 to give 6.4 parts by weight of citric acid. Yield 114%)

EXAMPLE 3.

25 A culture of *Candida tropicalis* IFO-0589 is inoculated into 250 parts by volume of a sterilized aqueous medium composed of ammonium sulfate (0.4%), potassium dihydrogen phosphate (0.1%) magnesium sulfate heptahydrate (0.1%) ferric sulfate heptahydrate (0.001%), calcium chloride (0.01%), manganese sulfate tetrahydrate (0.001%), yeast extract (0.05%), n-tetradecane (5%) (pH 5.0), and incubated with agitation and aeration at 28°C for 8 days while the pH of the culture broth is kept at 5.0 with the addition of sodium hydroxide, at the end of the incubation period 16.7 mg. of citric acid per ml. of the broth are accumulated.

EXAMPLE 4.

40 A culture of *Candida intermedia* IFO-0761 is inoculated into 250 parts by volume of a sterilized aqueous medium with a pH of 6.8 composed of ammonium sulfate (0.4%), potassium dihydrogen phosphate (0.05%), magnesium sulfate heptahydrate (0.05%), yeast extract (0.1%) calcium carbonate (3%) and distilled components including 82% n-paraffin with from 12 to 16 carbon atoms (5%), and incubated under agitation and aeration at 30°C for 6 days, at the end of the incubation period, 12.4 mg. of citric acid per ml. of the culture broth are accumulated.

EXAMPLE 5.

55 A culture of *Candida parapsilosis* IFO-0708 is inoculated into 250 parts by volume of a sterilized medium with the pH of 7.2 composed of the same components as in Example 1 plus calcium acetate (1%) and 10 parts by weight of distilled fraction including n-paraffin with from 16 to 20 carbon atoms,

and is incubated under agitation and aeration for 8 days, at the end of the incubation period, 18.1 mg. of citric acid per ml. of culture broth are accumulated.

EXAMPLE 6.

65 In the same manner as in Example 2, employing a medium as described in Example 2, 18.4 mg. of citric acid per ml. of the culture broth are accumulated by the incubation of *Candida guilliermondii* IFO-0838.

WHAT WE CLAIM IS:—

70 1. A method of producing citric acid which comprises inoculating a citric acid-accumulating and hydrocarbon-assimilating strain of a yeast belonging to the genus *Candida* in an aqueous culture medium containing at least one of the normal paraffins with from 9 to 20 carbon atoms as the main carbon source; incubating the culture at a pH of from 4 to 7.5 until citric acid is accumulated in the culture broth; and recovering the so-accumulated citric acid therefrom.

80 2. A method according to claim 1, wherein the incubation temperature is from 15° to 40°C.

85 3. A method according to claim 1 or 2, wherein the pH of the culture medium is kept from 5 to 6.5.

90 4. A method according to any of claims 1 to 3, wherein the normal paraffins are used in an amount of from 3 to 20% by volume of the culture medium.

95 5. A method according to any of claims 1 to 4, wherein the yeast is *Candida Lipolytica*.

6. A method according to any of claims 1 to 4, wherein the yeast is *Candida tropicalis*.

100 7. A method according to any of claims 1 to 4, wherein the yeast is *Candida intermedia*.

8. A method according to any of claims 1 to 4, wherein the yeast is *Candida parapsilosis*.

105 9. A method according to any of claims 1 to 4, wherein the yeast is *Candida guilliermondii*.

10. A method according to claim 5, wherein the yeast is *Candida lipolytica* IFO-1437.

11. A method according to claim 6, wherein the yeast is *Candida tropicalis* IFO-0589.

110 12. A method according to claim 7, wherein the yeast is *Candida intermedia* IFO-0761.

13. A method according to claim 8, wherein the yeast is *Candida parapsilosis* IFO-0708.

115 14. A method according to claim 9, wherein the yeast is *Candida guilliermondii* IFO-0838.



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15. A method for producing citric acid substantially as herein described with reference to any of the specific examples.

5 16. Citric acid when produced by a method as claimed in any of claims 1 to 15.

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