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[54] **CULTURE MEDIUM AND PROCESS FOR THE BIOLOGICAL PRODUCTION OF ALPHA, OMEGA-ALKANEDIOIC ACID**

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[51] **Int. Cl.** **C12b 1/00**

[58] **Field of Search** ..... 195/114, 3 H, 28 R, 195/29, 30, 47, 100

[56] **References Cited**

OTHER PUBLICATIONS

A Compilation of Culture Media for Cultivation of Microorganisms, M. Levine & H. W. Schoenlein, Williams & Wilkins Co., 1930, pp. 31 & 32.

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[57] **ABSTRACT**

An aqueous culture medium comprising (1) 3 to 35 grams per liter of alkali metal, calcium nitrate, magnesium nitrate, strontium nitrate and/or beryllium nitrate; (2) 0.3 to 10 grams per liter of alkali metal phosphate; (3) 1 to 15 grams per liter of an alkali metal acetate, calcium acetate, magnesium acetate, strontium acetate and/or beryllium acetate; and (4) a nutrient source; and a process for the production of an alpha,omega-alkanedioic acid of 10 to 16 carbon atoms comprising (1) introducing a mutated strain of Corynebacterium into a composition which has a pH of from 6 to 9 and which comprises 1 to 50 percent by volume of an n-alkane of 10 to 16 carbon atoms and a complemental amount of the culture medium above; (2) maintaining the composition at a temperature between 20° and 45°C. for 48 t0 96 hours; and (3) separating the alpha,omega-alkanedioic acid from the resulting mixture.

5 Claims, No Drawings

CULTURE MEDIUM AND PROCESS FOR THE BIOLOGICAL PRODUCTION OF ALPHA, OMEGA-ALKANEDIOIC ACID

This invention relates to the preparation of alpha,omega-alkanedioic acid from n-alkanes through the use of bacteria. Particularly, this invention relates to the preparation of alpha,omega-alkanedioic acid of 10 to 16 carbon atoms from n-alkanes of 10 to 16 carbon atoms by the use of a mutant of Corynebacterium. More particularly, this invention relates to a culture medium for use in the preparation of alpha,omega-alkanedioic acid from n-alkanes of 10 to 16 carbon atoms and a mutant of Corynebacterium and an improved process which utilizes the culture medium in the preparation of alpha,omega-alkanedioic acid of 10 to 16 carbon atoms.

It was known that long chain alkanes could be oxidized utilizing *Corynebacterium* 7EIC (ATCC–19067) to form their respective acids. However, only low yields and conversions were obtained in such processes. Culture media, of course, were utilized but none of these allowed for high yields and conversions of the alkanes to their respective alpha,omega-alkanedioic acid. Therefore, an improved culture medium which would allow for such improved yields and conversions was sought. An improved process was also sought to further improve the yields and conversions of the alkanes to the alpha,omega-alkanedioic acid.

Such a culture medium has been found. It is aqueous and comprises (1) 3 to 35 grams per liter of a member selected from the class consisting of alkali metal nitrate, calcium nitrate, magnesium nitrate, strontium nitrate, beryllium nitrate and mixtures thereof; (2) a 0.3 to 10 grams per liter of alkali metal phosphate; (3) 1 to 15 grams per liter of an alkali metal acetate, calcium acetate, magnesium acetate, strontium acetate, beryllium acetate and mixtures thereof; and (4) a nutrient source.

The improved process for the production of an alpha,omega-alkanedioic acid of 10 to 16 carbon atoms comprises (1) introducing a mutated strain of Corynebacterium capable of producing at high yield and conversion alpha,-omega-alkanedioic acid of 10 to 16 carbon atoms into a composition which has a pH of from 6 to 9 and which comprises 1 to 50 percent by volume of an n-alkane of 10 to 16 carbon atoms and a complemental amount of the culture medium above; (2) maintaining the composition at a temperature of between about 20° to 45°C. for 48 to 96 hours; and (3) separating the alpha,omega-alkanedioic acid from the resulting mixture.

Some preferred mutated strains of the *Corynebacterium* are on file with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852. These strains are referred to throughout by their ATCC number. The organism tentatively assigned to the genus *Corynebacterium* was first isolated by J. W. Foster (J. Bacteriology 85, 859 (1963). The original culture was pink but after a series of mutations with chemical and physical mutagens the culture lost the capability of producing a pigment. The organism is a non-motile, gram (+) rod with metachromatic granules. Snapping division produces angular and palisade arrangement of cells. Older cells often lose gram (+) characteristic with granules still evident. The bacterium is an obligate aerobe and utilizes nitrates as sole source of nitrogen. The bacterium rods are 0.3 to 0.4 by 0.7 to 0.8 micron. They grow well on most common laboratory media, but grow better on the culture medium of this invention. Colonies of bacteria on tryptone glucose agar are dry, granular and small. Optimum temperature of growth is 37°C.

The amount of metal nitrate utilized in the culture medium depends on the mutant of *Corynebacterium* which is used. A relatively large amount of nitrate is required for the bacterium utilizes it as the sole source of nitrogen. Preferably 6 to 30 grams per liter of the metal nitrate is present in the culture medium. The preferred nitrates are the alkali metal nitrates, in particular sodium or potassium. The most preferred concentration of the metal nitrate in the culture medium is 12 to 25 grams per liter.

The alkali metal phosphate is used as a buffer component in the culture medium. A sufficient amount of the phosphate is present in the culture medium to cause it to have a pH of from 6 to 9. Therefore, the amount utilized will vary according to the other components in the culture medium. The preferred amount of alkali metal phosphate in the culture medium is 1 to 6 grams per liter. Preferred phosphates are K_2HOP_4 and $NaH_2PO_4 \cdot H_2O$.

The metal acetate is critical as a component in the culture medium in that it significantly increases the yield of the alpha,omega-alkanedioic acid when the culture medium is used in the process of this invention. The preferred concentration for the metal acetate is from 2.5 to about 10 grams per liter. Preferred acetates are the alkali metal acetates, in particular, sodium and potassium.

The type of nutrient source in the culture medium is not critical. Examples of useful nutrient sources are tomato juice, broth solids, molasses plus yeast extract or cottonseed meal. The first two types of nutrient source are preferred. The amount of nutrient source utilized is normally from 1 to 5 grams per liter, preferably around about 2 grams per liter of the culture medium.

The balance of the culture medium is made up of water. This can be tap water or distilled water. Other components can be present in the culture medium if they do not impede the function of the main components. Some of such components are $MgSO_4 \cdot 7H_2O$, $CaCl_2$, (neither of these two should be used in large amounts or they made cause the phosphate to precipitate), $FeSO_4$ with ethylenediamine tetraacetic acid chelating agent, $MnSO_4 \cdot H_2O$, H_3BO_3, $Na_2MoO_4 \cdot 2H_2O$, KI, and $ZnSO_4 \cdot 7H_2O$. It is preferred that no ammonium compounds be present for they erode the ability of the medium to cause the bacterium to accumulate acid. Copper compounds can be included in the medium. However, they tend to give no advantage but instead result in reduced acid production

The culture medium can contain emulsifier. This is not required but does normally render the culture medium more conducive to manipulation. Useful emulsifiers are substances such as "Ethofats" (Armour and Company, Chicago, Illinois) which is monofatty or rosin acid esters of polyoxyethylene glycols having the general formula

$$RC(=O)O(CH_2CH_2O)_xH.$$

Examples of other nutrient type additives which can be included in the culture medium are amino acids, vitamins, glucose, sugar, adipic acid, butyric acid and glutaric acid.

The culture medium which is discussed above is the medium which is used in the process for preparing the alpha,omega-alkanedioic acid in the full scale fermentation but is normally not used in the pregrowth of the bacteria. Pregrowth of the bacteria can be accomplished by placing a lyophile culture of freeze-dried bacteria on a tomato juice agar slant which contains proteins and carbohydrates, i.e., tomato juice extract and glucose. The bacteria grows for from 24 to 48 hours at 37°C. on the slant. A loopful of the bacteria is then transferred to a large flask such as a 125 ml. Erlenmyer for pregrowth. In the pregrowth stage the bacteria normally grows on a solution comprising 5 weight-/volume percent molasses, preferably cane, 0.5 weight-/volume percent yeast extract, 0.15 weight/volume percent K_2HPO_4, 0.06 weight/volume percent $NaH_2PO_4 \cdot H_2O$ and water. Weight/volume percent means grams/-milliliters in percent terms, i.e., 5 grams in 100 mls. is 5 weight/volume percent.

The pregrowth flask is maintained at about 37°C. and vibrated at 220 rpms on a shaker with a 1 inch throw for 24 hours. The volume of the material in the initial pregrowth flask is normally 25 ml. although this is not critical. The pregrowth medium plus bacteria are built up by successive spiking of larger and larger volumes. When a pregrowth volume is obtained which is about 10 percent of the total volume of materials to be used to make the alpha, omega-alkanedioic acid, the pregrowth stage is completed. At this point the pregrowth medium should contain at least 1×10^9 organisms per ml. Normally, all the pregrowth medium is used to inoculate the culture medium and n-alkane.

The culture medium described above is useful in growing bacteria which produce acids from hydrocarbons. In particular, the culture medium is useful in preparing alpha,omega-alkanedioic acids from n-alkanes of 10 to 16 carbon atoms by using mutants of *Corynebacterium*. The process produces higher levels of conversion and yield on the n-alkanes of 10 to 14 carbon atoms. It is most useful on dodecane which produces 1,12-dodecanedioic acid. The n-alkane utilized can be one n-alkane or a mixture of such. A mixture of acids then can be produced by the process of this invention.

In the recitation of the process above it is stated that the *corynebacterium* mutant is capable of producing at high yields and conversions the alpha,omega-alkanedioic acid. When the process of this invention is utilized, this statement means to produce alpha,omega-alkanedioic acid at yields and conversions so that the percentage of the n-alkane converted to the alpha,omega-alkanedioic acid is at least 6.8 mole percent; preferably at least 10 mole percent. This is calculated by multiplying the yield times the conversion. Conversion is defined as the percentage amount in moles of the n-alkane converted to other than the n-alkane without consideration as to what it is converted to while yield means the percentage amount in moles of the n-alkane converted which is in the form of the alpha,omega-alkanedioic acid. Normally, when conversion is high, yield is lower and vice versa. Therefore, these can vary up and down but the product of the two is always greater than the above recited percentages.

The composition inoculated by the bacteria in the process normally comprises 1 to 50 percent by volume of the n-alkane with 5 to 20 percent by volume being preferred. The remainder of the composition is the culture medium as discussed above. Normally, the Corynebacterium mutant is added to the composition as part of an inoculum. This inoculum usually represents about 5 to 20 percent by volume of the composition plus the inoculum.

The pH of the composition, i.e., the culture medium and n-alkane, should be from 6 to 9 initially with the preferred range being from 7 to 8. The most preferred range is from 7 to 7.5. The temperature of the composition should be from 20° to 45°C. both at the time the bacterium is added and during fermentation. The preferred range is 25° to 35°C. Contact of the bacteria and the composition is for up to 96 hours. Higher yields and conversions are not obtained until about 48 hours and after 72 hours diminishing returns set in.

One method of separating the alpha,omega-alkanedioic acid from the resulting mixture is to (1) remove the cells and excess hydrocarbon from the aqueous phase by phase separation, (2) adjust the pH of the aqueous layer to 2.0 with concentrated sulfuric acid, (3) filter out the precipitate (alpha,omega-alkanedioic acid), and (4) wash the precipitate with water. The purity of the acid is normally 50 to 65 percent by weight when this procedure is followed.

Mutants of the *Corynebacterium* which are useful in the process can be prepared by the standard techniques of (1) high energy radiation; (2) chemical mutation; (3) lethal synthesis and combinations thereof. The two former techniques are preferred. The mutants referred to herein were prepared by one or a combination of the three methods.

The culture medium and process are useful in preparing the alpha,omega-alkanedioic acid. The acids are useful as intermediates in the preparation of polyamides which have low water absorption and good stiffness and which can be formed into fibers.

The following Examples are meant to illustrate but not to limit the invention. Parts and percentages are by weight unless otherwise specified.

EXAMPLE I

Freeze-dried mutant of *Corynebacterium sp.* G 11–25, ATCC 21744 was grown on a tomato juice agar slant. A loopful of the Corynebacterium was transferred to 50 mls. of molasses-yeast broth in a 300 ml. shake flask (Erlenmyer). The broth had been previously autoclaved for 15 minutes at 121°C. The composition of the molasses-yeast broth was as follows:

0.15 weight/volume percent K_2HPO_4,
0.06 weight/volume percent $NaH_2PO_4 \cdot H_2O$,
5 weight/volume percent cane molasses,
0.5 weight/volume percent yeast extract, and
remainder - water.

Its pH had been adjusted to 7.0 to 7.1 with NaOH. The broth containing the Corynebacterium mutant was then incubated for 24 hours at 37°C. in a 1 inch throw rotary shaker at 220 rpms. 25 Mls. of this broth was then transferred to 250 mls. of fresh molasses-yeast broth prepared as above in a 1 liter shaker flask. This broth was incubated for 24 hours at 37°C. in a 1 inch throw rotary shaker at 240 rpms. This broth was then used as an inoculum for the main fermentation media test flasks.

The main fermentation test flasks were prepared as follows:

1. A salts and buffer medium was prepared in distilled water and contained the following ingredients in their respective concentrations:
   0.04 weight/volume percent $MgSO_4 \cdot 7H_2O$
   0.3) weight/volume percent K_2HOP_4
   0.12 weight/volume percent $NaH_2PO_4 \cdot H_2O$
   5 weight/volume percent $NaNO_3$
   0.003 weight/volume percent $CaCl_2$

	Stock Solution Concentration
0.2 volume percent of stock solution $FeSO_4$ - ethylene-diamine tetraacetic acid	0.125 grams/100 mls.
0.2 volume percent of stock solution $MnSO_4 \cdot H_2O$	80 mgs/100 mls.
0.2 volume percent of stock solution H_3BO_3	5 mgs/100 mls.
0.2 volume percent of stock solution $Na_2MoO_4 \cdot 2H_2O$	43 mgs/100 mls.
0.2 volume percent of stock solution KI	20 mgs/100 mls.
0.2 volume percent of stock solution $ZnSO_4 \cdot 7H_2O$	80 mgs/100 mls.

The pH of the salts and buffer medium was adjusted to 7.1 to 7.2 with NaOH.

2. The salts and buffer medium was diluted with distilled water to 65 percent of its original concentration. To the diluted salts and buffer medium was added 0.3 weight/volume percent tomato juice and 0.5 weight/volume percent sodium acetate. 20 Mls. of this solution were added into a 300 ml. shaker flask with 0.1 ml. "Ethofats" (mono-fatty or rosin acid esters of polyoxyethylene glycols) to form the culture medium. 2.5 Mls. dodecane were also added to the shaker flask. The flasks were gauze covered and auto-claved.

Into the main fermentation test flasts prepared above were added 5 mls. of the inoculum broth prepared as above. The flasks were then incubated at 30°C. for three days in a rotary shaker at 220 rpms. with a 1 inch throw.

After the incubation period, all flasks were adjusted to the initial volume with distilled water. 10 Ml. samples were centrifuged for separation of the residual hydrocarbon, cells, and aqueous fractions. The cells settled between the oil and aqueous phases. The 1,12-dodecanedioic acid assay of the aqueous layer was done by thin layer chromatography. Twenty-two millimoles of 1,12-dodecanedioic acid were produced per liter of the materials in the fermentation test flasks at the end of the fermentation. The percent (mole) yield was 38 and the percent (mole) conversion was 18.

EXAMPLE II

Following the procedure of Example I, 1,12-dodecanedioic acid was produced from n-dodecane from a *Corynebacterium sp.* G 11–25, ATCC 21744. The amounts of sodium nitrate and sodium acetate were varied in the culture medium as shown in Table I. The results of the acid analysis are also shown. When the nitrate was being varied, the amount of sodium acetate was 0.50 weight/volume percent while when the acetate was being varied, the amount of sodium nitrate was 2.50 weight/volume percent.

TABLE I

MEDIA VARIABLES	1,12-Dodecanedioic acid mM/ liter	% Yield (mole)	% Conversion (mole)
1. $\%NaNO_3$=0.313% (weight/volume)	38	18	72
2. $\%NaNO_3$=0.625% (weight/volume)	38	16	84
3. $\%NaNO_3$=1.25% (weight/volume)	40	18	76
4. $\%NaNO_3$=2.50% (weight/volume)	36	21	62
5. $\%NaC_2H_3O_2$=0.0% (standard)	12	20	20
6. $\%NaC_2H_3O_2$=0.25% (weight/volume)	31	22	50
7. $\%NaC_2H_3O_2$=0.50% (weight/volume)	34	25	54
8. $\%NaC_2H_3O_2$=1.00% (weight/volume)	20	21	36

mM = millimoles

EXAMPLE III

Following the procedure of Example I, 1,12-dodecanedioic acid was prepared from dodecane using the various *Corynebacterium* strains shown in Table II. The weight/volume percent of sodium nitrate utilized was 2.5 rather than that of Example I. The results of the run are shown in Table II.

TABLE II

STRAIN	1,12-DODECANEDIOIC ACID mM/ Liter	% Yield (mole)	% Conversion (mole)
Corynebacterium sp. G 11-25, ATCC 21744	46	40	41
	37	34	36
	41	28	49
	37	46	28
	41	36	45
	42	32	36

TABLE II-Continued

STRAIN	1,12-DODECANEDIOIC ACID mM/ Liter	% Yield (mole)	% Conversion (mole)
Corynebacterium sp. G17 B5, ATCC 21747	30	11	100
	44	17	100
	46	19	100
	58	23	36
Corynebacterium sp. 234-30, ATCC 21745	42	52	32
	48	33	41
	31	32	29
	97	31	92
	21	35	21
Corynebacterium sp. G 11-32, ATCC 21746	51	30	65
	58	24	37
	53	28	68
	69	30	63
	72	30	70
	34	47	26

mM = millimoles

We claim:

1. A process for the production of an alpha, omega-alkanedioic acid of 10 to 16 carbon atoms which comprises (1) introducing a mutated strain of Corynebacterium capable of producing at high yield and conversion alpha, omega-alkanedioic acid of 10 to 16 carbon atoms into a composition which has a pH of from 6 to 9 and which comprises 1 to 50 percent by volume of n-alkane of 10 to 16 carbon atoms and from 50 to 99 percent by volume of an aqueous culture medium comprising (a) 3 to 35 grams per liter of a member selected from the group consisting of alkali metal nitrate, calcium nitrate, magnesium nitrate, strontium nitrate, beryllium nitrate and mixtures thereof, (b) 0.3 to 10 grams per liter of alkali metal phosphate, (c) 1 to 15 grams per liter of a member selected from the group consisting of alkali metal acetate, calcium acetate, magnesium acetate, strontium acetate, beryllium acetate and mixtures thereof and (d) a nutrient source; (2) maintaining the composition at a temperature of between about 20°C. and 45°C. for 48 to 96 hours and (3) separating the alpha, omega-alkanedioic acid from the resulting mixture.

2. The process of claim **1** wherein the n-alkane is from 10 to 14 carbon atoms, the amount of n-alkane in the composition is 5 to 20 percent by volume, and the amount of culture medium is 80 to 95 percent by volume.

3. A process for the production of an alpha, omega-alkanedioic acid of 10 to 16 carbon atoms which comprises (1) introducing a mutated strain of Corynebacterium capable of producing at high yield and conversion alpha, omega-alkanedioic acid of 10 to 16 carbon atoms into a composition which has a pH of from 6 to 9 and which comprises 1 to 50 percent by volume of n-alkane of 10 to 16 carbon atoms and from 50 to 99 percent by volume of a culture medium comprising (a) 6 to 30 grams per liter of a member of the group consisting of sodium nitrate and potassium nitrate, (b) 1 to 6 grams per liter of an alkali metal phosphate, (c) 2.5 to 10 grams per liter of a member selected from the group consisting of sodium acetate and potassium acetate and (d) a nutrient source; (2) maintaining the composition at a temperature of between about 20°C. and 45°C. for 48 to 96 hours and (3) separating the alpha, omega-alkanedioic acid from the resulting mixture.

4. The process of claim **3** wherein the n-alkane is from 10 to 14 carbon atoms, the volume of the n-alkane in the composition is 5 to 20 percent by volume, and the amount of culture medium is 80 to 95 percent by volume.

5. The process of claim **4** wherein the n-alkane contains 12 carbon atoms.

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