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PRODUCTION OF GLUTAMIC ACID

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9 Claims. (Cl. 195—1)

This application is a division of our copending application Serial No. 308,637, filed September 13, 1963, now abandoned.

This invention relates to the oxidation of hydrocarbons. More particularly, this invention relates to the fermentation of hydrocarbons to produce L-glutamic acid. Still more particularly, it is concerned with the conversion of hydrocarbons to L-glutamic acid in the free state, and to the substantial exclusion of D-glutamic acid by the fermentation of hydrocarbons with a certain strain of glutamic acid-producing microorganism.

The preparation of L-glutamic acid by fermentation processes is well known in the prior art. In each of these processes, however, there has been employed as the sole source of carbon and energy a carbohydrate or other complex oxygenated organic compound. Such a source of energy in a fermentation process is disadvantageous because substrates of this sort, and particularly carbohydrates, are relatively expensive raw materials.

Therefore, it is an object of this invention to provide a process for the production of L-glutamic acid utilizing an inexpensive substrate which can readily be converted to L-glutamic acid in high yield. A further object is to produce L-glutamic acid without the concurrent formation of D-glutamic acid, thus avoiding the problems of isolation and separation of L-glutamic acid from a racemic mixture.

It has now been found, in accordance with the present invention, that these objects can be achieved by growing the hereinafter specified microorganism on inexpensive hydrocarbon substrates as essentially the sole carbon source to yield L-glutamic acid. When such a fermentation is carried out under the conditions and with the microorganism described in detail below, L-glutamic acid is produced in high yield substantially free of D-glutamic acid. That hydrocarbon substrates can be employed in this manner is particularly surprising since most work with hydrocarbons heretofore has resulted in the formation of carbon dioxide and water as the principal fermentation products rather than commercially valuable oxygenated organic compounds.

The microorganism employed in accordance with this invention belongs to the genus *Nocardia* and the species *globetula* but cannot be identified as being the same as any strain described in Bergey's Manual of Determinative Bacteriology, 7th Edition. This microorganism therefore is considered to be a new strain, and a culture of this new strain has been deposited in the American Type Culture Collection in Washington, D.C., and is designated as *Nocardia globetula* ATCC 15076. This microorganism, as has now been discovered, has an outstanding ability to convert hydrocarbons to L-glutamic acid in high yield. While various other microorganisms can utilize hydrocarbons as their source of carbon and

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to some extent produce L-glutamic acid in the fermentation, none has been found that is comparable to this new strain *Nocardia globetula* with respect to the production of this particular amino acid.

Nocardia globetula ATCC 15076 possesses the following cultural characteristics:

- (1) Gram stain—gram variable, long rods to cocci, chains
- (2) Methylene blue stain—granules, long rods to cocci
- (3) Acid fast stain—not acid fast, rods to cocci
- (4) Kligler iron agar—good growth, moist, raised cream colored
- (5) Methyl red—Voges Proskauer—both negative
- (6) Gelatin liquefaction—good growth, no liquefaction
- (7) Nitrate broth—nitrite negative
- (8) Colony description—nutrient agar—cream colored, smooth, raised, moist
- (9) Litmus milk—sediment, no noticeable change
- (10) Loeffler's blood serum—good growth, flesh colored, raised, smooth, moist
- (11) Dorset egg medium—luxurious growth, smooth, moist, orange
- (12) Sim's medium—no H₂S, Indole negative
- (13) Glycerol agar—good growth, small cream colored colonies
- (14) Starch agar—good growth, white, smooth, raised, doesn't hydrolyze starch
- (15) Potato dextrose agar—scant growth, raised tan, moist
- (16) Carbohydrate test in phenol red broth using 0.5% specific carbohydrate substrate
 - (a) Mannitol—no acid, no gas
 - (b) Levulose—no acid, no gas
 - (c) Lactose—no acid, no gas
 - (d) Inositol—no acid, no gas
 - (e) Saccharose—no acid, no gas
 - (f) Arabinose—no acid, no gas
 - (g) Maltose—no acid, no gas
 - (h) Dextrose—no acid, no gas

The nutrient medium employed for the culture of the aforesaid species of this invention to produce L-glutamic acid can vary considerably, but should contain, in addition to the hydrocarbon substrate as essentially the sole source of carbon, a source of nitrogen and salts.

The type of hydrocarbon substrate employed can vary widely ranging from lower alkyl compounds such as methane or ethane to substituted aromatics such as dimethylnaphthalenes. Where the hydrocarbons are volatile, it is preferred to use a closed system to insure maximum utilization of the substrate by the microorganism and to prevent loss of the volatile substrate into the atmosphere. Other specific hydrocarbons which have been found to be useful in this process are such compounds as tetrahydronaphthalene, dodecylbenzene, decane, n-hexadecane, and the like. Mixtures of these and other hydrocarbons are likewise suitable. Thus, generally speaking, any refinery cuts which contain large amounts of normal paraffins in the C₁₀–C₃₀ range, such as Bahia gas oil or fuel oil No. 2 can be employed as substrates in this invention.

The quantity of hydrocarbon substrate employed in this process may range from 1 to 8% of the total nutrient medium and preferably should be from 3 to 6%. It is desirable in carrying out this process that the hydrocarbon substrate be introduced into the fermentation

broth in small increments so that the hydrocarbon is present in the nutrient medium in amounts below growth-limiting concentrations. Thus, for example, where small amounts of hydrocarbon are being employed, it has been found preferable to introduce at the beginning of the fermentation, as little as 0.25% of the total hydrocarbon to be added, then, after twelve hours, an additional 0.10%, while after twenty-four hours an additional 1% may be introduced into the fermentation broth. Depending upon the total amount of hydrocarbon to be utilized, proportionately larger amounts may be added at twelve to twenty-four hour periods thereafter until the fermentation is complete.

Examples of suitable nitrogen sources are urea, soybean meal, ammonium salts such as ammonium sulfate, ammonium phosphate and the like. Other nitrogen sources can be used but are somewhat less effective. Care must be exercised in maintaining a suitable pH when ammonia or ammonium salts are used as nitrogen sources. Mixtures of two or more of these materials can also be used. The concentration of suitable nitrogen source material in the nutrient medium is desirably from about 0.5 to 2%.

The character of the mineral salts used in the nutrient medium can vary to some extent but in any event the nutrient medium should contain substantial amounts of phosphorus and magnesium salts in addition to a nitrogen source as mentioned above. Additionally certain trace mineral salts should be present, and a suitable mineral salts composition for this purpose is as follows:

Compound:	μg./liter
FeSO ₄ ·7H ₂ O -----	1000
CuSO ₄ ·5H ₂ O -----	197
H ₃ BO ₃ -----	57
MnSO ₄ ·H ₂ O -----	31
ZnSO ₄ ·H ₂ O -----	193
Na ₂ MoO ₄ ·2H ₂ O -----	35
CoCl ₂ ·6H ₂ O -----	100
NiCl ₂ ·6H ₂ O -----	5
CaCl ₂ -----	1000

In addition to the foregoing constituents of the nutrient medium, a source of growth stimulating materials can be used at a concentration of from about 0.01 to 0.1%. Material such as distillers solubles, yeast extract and other substances of this nature well known in the fermentation field are especially effective.

The process is desirably carried out at a pH of from about 6.0 to 8.0 and preferably at a pH of from 7.4 to 8.0. As the culture grows and glutamic acid is produced, the medium tends to become more acidic. Therefore, it may be necessary to adjust the pH of the fermentation medium with a base such as ammonium hydroxide periodically. Alternatively, buffering materials can be used, in which case they can be conveniently added to the nutrient medium prior to fermentation by diluting the ingredients with an aqueous solution of alkali metal hydrogen phosphates to obtain the desired volume of fermentation broth. One such suitable buffering solution having a pH of 7.4 can be prepared by mixing an aqueous solution of 11.88 grams of Na₂HPO₄·2H₂O per liter of water and a solution of 9.08 grams of KH₂PO₄ per liter of water, and mixing them in a 4:1 ratio.

The culturing of the microorganisms in accordance with the process of this invention is carried out under aerobic conditions. While sufficient aeration is generally obtained in small flasks by mechanical agitation during the fermentation period, it is important in larger scale operations that the broth be stirred mechanically and that sterile air be introduced into the vessels by known methods. It has been generally found desirable to introduce from about one-half to one volume of air per minute for each volume of fermentation broth. For optimum yields of L-glutamic acid, fermentation with the microorganism employed in accordance with this invention is conducted at about 25-

34° C., and preferably at about 30° C. for from 48 to 96 hours. Typical yields of L-glutamic acid after about 72 to 96 hours of incubation of the organism *Nocardia glob-
erula* ATCC 15076 under the above conditions are 1-4 grams per liter.

The microorganism inoculum is desirably prepared by introducing a 2 ml. wash from a culture slant into 100 ml. of a complex carbohydrate nutrient medium contained in a 500 ml. flask where it is grown for 16 to 24 hours. A 5 to 10% inoculum is then obtained from this culture broth by introducing 5 to 10 parts of this broth into 100 parts of mineral salts-hydrocarbon fermentation medium described above.

The L-glutamic acid produced in accordance with this process can be conveniently recovered from the fermentation broth by several methods known in the fermentation art; as, for example, by first separating the cells and other solid matter by filtration or centrifugation, and thereafter acidifying the fermentation liquor with a mineral acid such as hydrochloric acid, followed by adsorption of the L-glutamic acid on a weakly basic anion exchange resin such as Rohm & Haas Company's Amberlite IR-4B. The amino acid is then removed from this resin by elution with dilute hydrochloric acid, and concentration of the eluate to a small volume. L-glutamic acid hydrochloride can then be obtained from this solution by known crystallization methods.

Alternatively, since the microorganism itself will contain a high proportion of glutamic acid in combined form, the whole broth containing the microorganism first can be treated with an equal volume of a strong mineral acid such as hydrochloric acid. This acidified broth is then heated for several hours at about 100° C., preferably under pressure, and the released L-glutamic acid is recovered by passage of the filtered broth over a column of acid-washed alumina. The alumina can then be eluted with a suitable solvent such as a dilute alkali to obtain the L-glutamic acid in purified form following concentration and crystallization of the eluate.

The amount of L-glutamic acid in a particular fermentation broth can be determined not only by actual isolation of the amino acid in the manner described above, but also by chemical and bio-assay methods. These assay methods are set forth in detail below:

1. *Enzymatic method.*—The procedure employed is based on that described in *Agricultural and Food Chemistry*, vol. 5, No. 6, pp. 448-457 (1957), wherein dried dead cells of *E. coli* suspended in a solution buffered at pH 5.0 are used as a reagent. This suspension contains enzymes, one of which quantitatively decarboxylates L-glutamic acid to γ-amino-butyric acid, and another of which hydrolyzes L-glutamic acid followed by decarboxylation. The enzyme liberates one mole of carbon dioxide from one mole of L-glutamic acid. Pyridoxal phosphate functions as the coenzyme of L-glutamic acid decarboxylase. From 1.5 to 2.5 mg. of L-glutamic acid can be measured manometrically in the Warburg apparatus with a precision within 1% in accordance with this procedure.

2. *Chemical methods.*—This method is based on the procedure described in U.S. Patent No. 3,032,474 wherein culture filtrates containing glutamic acid are spotted on Whatman No. 1 filter paper, gauging the volume applied so as to contain 10 to 90 mg. glutamic acid. A standard glutamic acid solution is spotted on the same sheet of paper at levels of 10, 30, 50, 70 and 90 mg. to enable constitution of a calibration curve. Unknowns and standard glutamic acid solutions are run in duplicates on different sheets of paper. The papers are allowed to develop for 20 to 24 hours by the descending method. Phenol saturated with an aqueous solution containing 6.3% sodium citrate and 3.7% monobasic sodium phosphate is used as the developing solvent. It is found to give a good separation of glutamic acid spots from other ninhydrin reacting spots present in the filtrates.

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Following overnight drying in a current of air, the chromatograms are sprayed lightly with a solution of 0.1% ninhydrin in water-saturated n-butanol. The glutamic acid spots are localized at R_f values of about 0.23 after heating the papers at 80–100° C. for about two minutes.

Quantitation of the glutamate in the spots is performed by the method of Smith and Agiza, Analyst, 76,623 (1951). Each glutamic acid spot is then cut into small pieces and placed in 1 ml. of water in a graduated test tube. Two ml. of citrate buffer solutions (pH 5) and 2 ml. of ninhydrin reagent are added in sequence to each tube. The tubes are placed in boiling water and 1 ml. of freshly prepared 0.2% SnCl₂ solution (in citrate buffer of pH 5) is added to each tube. After heating for 15 minutes, the tubes are removed and cooled in the dark for 10 minutes. The liquid in each tube is made up to 10 ml. by addition of a saturated NaCl solution. The color containing complex is then extracted from the aqueous phase by shaking with 5 ml. of n-butanol. The clear butanol solution is pipetted into a Klett tube and the intensity of the purple color is measured by a Klett-Summerson photoelectrocolorimeter, using a green filter. After correction for the reading of a blank prepared from the chromatogram but containing no glutamic acid, a straight line relationship is found to exist between color intensity and concentration of glutamic acid over a range of 10 to 90 mg. The glutamic acid content of the unknown filtrates is calculated from their respective standard calibration curves.

The following examples are specific illustrations of the invention:

Examples

A series of four fermentations was carried out in 500 ml. sterilized dispo-plugged Erlenmeyer flasks, using a sterile nutrient medium of the following composition:

Compound:	Concentration	
MgSO ₄	mg./liter..	400
(NH ₄) ₂ SO ₄	mg./liter..	3000
(Na) ₂ HPO ₄	mg./liter..	3000
FeSO ₄ ·7H ₂ O	μg./liter..	1000
CuSO ₄ ·5H ₂ O	μg./liter..	197
H ₃ BO ₃	μg./liter..	57
MnSO ₄ ·H ₂ O	μg./liter..	31
ZnSO ₄ ·H ₂ O	μg./liter..	193
Na ₂ MoO ₄ ·2H ₂ O	μg./liter..	35
CoCl ₂ ·6H ₂ O	μg./liter..	100
NiCl ₂ ·6H ₂ O	μg./liter..	5
CaCl ₂	μg./liter..	1000

The pH of the above medium was adjusted to 7.5 with NH₄OH, and 100 ml. of this medium were introduced into each flask. The medium was inoculated with 3% vegetative inoculum of *Nocardia globerula* ATCC 15076, which had been grown on decane for 36 hours, and the fermentation was conducted on a rotary mechanical shaker at 300 r.p.m. at 30° C. for 72 hours. The fermentation broth in each flask was periodically adjusted, as necessary, with additional amounts of concentrated NH₄OH in order to maintain the pH at 7.5. To each flask 8.8 mg. of decane substrate were added initially; 1760 mg. of decane were added after 24 hours and the same amount was add-

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ed after 48 hours (total 3%). The broth was harvested at 72 hours, at which time the bacterial cells and other impurities were removed by centrifugation and the amount of L-glutamic acid in the clear centrifugate was determined by the above-described enzymatic assay method. The following results were obtained:

Flask:	Free L-glutamic acid (gm./liter)
1	1.1
2	2.5
3	1.8
4	2.0

The invention claimed is:

1. A process for the production of L-glutamic acid which comprises subjecting a hydrocarbon to the action of *Nocardia globerula* ATCC 15076 under aerobic conditions, and recovering the resulting L-glutamic acid.

2. A process for the production of L-glutamic acid from hydrocarbons which comprises cultivating under aerobic conditions *Nocardia globerula* ATCC 15076 in a nutrient medium comprising a source of nitrogen and a hydrocarbon as the essential source of carbon, and recovering the resulting L-glutamic acid from the fermentation broth.

3. The method of synthesizing L-glutamic acid from hydrocarbons which comprises dispersing a hydrocarbon in an aqueous nutrient medium inoculated with *Nocardia globerula* ATCC 15076, subjecting the inoculated mixture to incubating conditions for a sustained period of time, and recovering L-glutamic acid from the resulting fermentation broth.

4. A process according to claim 1 wherein said conditions include a pH in the range of 6.0–8.0 and a temperature in the range of 25–34° C.

5. A process according to claim 4 wherein said hydrocarbon is a normal paraffin of the C₁₀–C₃₀ range.

6. A process according to claim 2 wherein said conditions include a pH in the range of 6.0–8.0 and a temperature in the range of 25–34° C.

7. A process according to claim 6 wherein said hydrocarbon is a normal paraffin of the C₁₀–C₃₀ range.

8. Method according to claim 3 wherein said conditions include a pH in the range of 6.0–8.0 and a temperature in the range of 25–34° C.

9. Method according to claim 8 wherein said hydrocarbon is a normal paraffin of the C₁₀–C₃₀ range.

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