

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO  
DIVISI3N DE NUEVAS CREACIONES

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Abogada  
JIMENA ESCOBAR URIBE

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|---------------------------------|-------------------------------------|-------------------------------|-------------------------------------|
| Patente de Invenci3n            | <input checked="" type="checkbox"/> | Patente de Modelo de Utilidad | <input type="checkbox"/>            |
| Vía Nacional                    | <input type="checkbox"/>            |                               |                                     |
| Vía PCT (fase nacional)         | <input checked="" type="checkbox"/> | Cap.I                         | <input type="checkbox"/>            |
|                                 |                                     | Cap. II                       | <input checked="" type="checkbox"/> |
| No. Sol. Internacional          | PCT/US2001/23149                    |                               |                                     |
| Fecha de Presentaci3n Internal. | 23/07/2001                          |                               |                                     |

Como resultado del examen de fondo, realizado con fundamento en el artícuo 45 de la Decisi3n 486 de la Comisi3n de la Comunidad Andina, se le comunica:

1. Documentos estudiados

- 1.1.    Descripci3n:                      Folios: 28 a 115 como se radic3 inicialmente.
- 1.2.    Reivindicaciones:           Folios: 116 a 121 como se radic3 inicialmente.  
                                                 Folios: 199 a 203 modificadas
- 1.3.    Figuras:                        Folios: 122 a 123 como se radic3 inicialmente.
- 1.4.    Prioridad:                        US 60/2220,794 del 25 de Julio de 2000

2. Objeto de la invenci3n

**Títuoo de la solicitud: "Métodos para incrementar la producci3n de etanol en una fermentaci3n microbiana"**

El objeto de la presente solicitud de patente se refiere a un método estable y continuo para producir etanol a partir de fermentaci3n de bacterias anaerobias de un sustrato gaseoso, el método comprende: cultivar bacterias anaeróbicas acetogénicas de Clostridium ljungdahlii en un bioreactor con un medio líquido en un pH de 5.5; suministrar a dicho reactor el sustrato gaseoso que comprende al menos un gas reductor seleccionado del grupo consistente de monóxido de carbono e hidrógeno; y manipular dichas bacterias reduciendo el potencial redox o incrementando la proporci3n de NAD(P)H a NAD(P) en el caldo de fermentaci3n después de que la bacteria alcanza una concentraci3n celular estable, estas condiciones de cultivo

incrementan la producción de etanol, quedando en una proporción etanol: acetato de 1:1 a 1:20.

El problema planteado en esta solicitud es la necesidad de incrementar la fermentación de bacterias acetogénicas de manera que se dirija la producción hacia la formación de etanol en lugar de acetato.

Para solucionar este problema técnico la solicitud en estudio proporciona un método estable y continuo en el cual se adecuan las condiciones de cultivo para que la fermentación se dirija a la producción de etanol, a partir de un sustrato gaseoso.

El objeto de la solicitud definida anteriormente se clasificó en la IPC C12P 7/06

3. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria – 25/07/2000

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

| Nº | Documento    | Título                                                                              | Fecha de publicación |
|----|--------------|-------------------------------------------------------------------------------------|----------------------|
| D1 | US 5,173,429 | "Clostridium ljungdahlii, an anaerobic ethanol and acetate producing microorganism" | Dic 22, 1992         |
| D2 | US 4,812,410 | "Continuous process for ethanol production by bacterial fermentation"               | Mar 14, 1989         |

4. Análisis de patentabilidad (artículo 14 D486)

4.1. Nivel Inventivo (artículo 18 D486)

1. Respecto a la solicitud en estudio:  
La solicitud en estudio reclama un método estable y continuo para producir etanol a partir de la fermentación anaeróbica bacteriana de un sustrato gaseoso, el método comprende: cultivar en un bioreactor bacterias de *Clostridium ljungdahlii*; suministrar sustrato gaseoso que tiene monóxido de carbono; alimentar pantotenato de calcio en dicho bioreactor, mantener la tasa de toma de CO en 0.3 mmoles de CO/ gramos de peso seco celular/minuto.
2. Respecto al estado de la técnica:  
D1 divulga la capacidad de producir etanol de la bacteria *Clostridium ljungdahlii*, detallando la forma en que se dirige la producción de etanol o de acetato de acuerdo con las condiciones del cultivo (resumen), en especial por mantener el pH entre 4.0 y 4.5 para favorecer la producción de etanol (columna 2, líneas 24 a 26). También divulga la habilidad de este microorganismo para fermentar etanol a partir de sustrato gaseoso tipo CO y H<sub>2</sub>O y/o CO<sub>2</sub> y H<sub>2</sub>(columna 2, línea 62 a 66).
- D2 divulga un proceso continuo para la producción de etanol por fermentación con cepas de Zynomonas, en este proceso se divulga la utilización de compuestos orgánicos tipo pantotenato de calcio para la formulación del medio de cultivo, de manera que se incremente y optimice la producción de etanol (columna 14, líneas 1 a 3; tabla 7).

3. Análisis de las diferencias con respecto al estado de la técnica:

| Características esenciales de la Solicitud                                                             | D1                            | D2                                     |
|--------------------------------------------------------------------------------------------------------|-------------------------------|----------------------------------------|
|                                                                                                        | US 5,173,429                  | US 4,812,410                           |
| Método continuo para producir etanol a partir de fermentación anaeróbica                               | SI (resumen)                  | SI (resumen)                           |
| Cultivar en un bioreactor bacterias de de <i>Clostridium ljungdahlii</i> en un medio líquido nutriente | SI (resumen)                  | NO                                     |
| Suministrar sustrato gaseoso que tiene monóxido de carbono                                             | SI (columna 2, línea 62 a 66) | NO                                     |
| Alimentar pantotenato de calcio en dicho bioreactor                                                    | NO                            | SI (columna 14, líneas 1 a 3; tabla 7) |
| Mantener la tasa de toma de CO en por lo menos 0.3 mmoles de CO/gr de peso seco de células/minuto      | SI (ejemplo 1)                | NO                                     |

El estado de la técnica más cercano es D1, el cual plantea un método para obtener etanol a partir de la misma especie bacterial utilizada en la solicitud, suministrando como fuente de carbono un sustrato gaseoso de CO y en donde además se establecen las condiciones para optimizar la producción de el etanol en cambio del acetato. La diferencia entre D1 y la solicitud es que esta última alimenta pantotenato de calcio al bioreactor como fuente orgánica de nutrientes en el medio de cultivo.

D2 justamente utiliza el pantotenato de calcio en el medio de cultivo para optimizar la fermentación y producción de etanol, en donde este compuesto es adicionado al medio de cultivo de la bacteria.

De esta manera la combinación de los documentos D1 y D2, le sugiere a una persona conocedora del arte la realización de un método de obtención de etanol a partir de bacterias anaeróbicas tipo *Clostridium ljungdahlii*, en las mismas condiciones establecidas en la solicitud. Por tanto, ante la información divulgada en D1 y D2 la invención carece de nivel inventivo.

De acuerdo con lo anterior la materia reclamada no cumplen con el requisito de nivel inventivo establecido en el artículo 18 de la Decisión 486.

5. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

| N° | Documento N° | Reivindicaciones afectadas | Requisito que afecta |
|----|--------------|----------------------------|----------------------|
| D1 | US 5,173,429 | 1-26                       | Nivel inventivo      |
| D2 | US 4,812,410 | 1-26                       | Nivel inventivo      |

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

2006

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.

**ALIX CARMENZA CÉSPEDES DE VERGEL**  
Jefe de División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha  
19 DIC. 2008

|                                     |                                               |
|-------------------------------------|-----------------------------------------------|
| <b>CONCEPTO TÉCNICO No.</b><br>3517 | <b>EXAMINADOR TÉCNICO</b><br>Código No.202052 |
|-------------------------------------|-----------------------------------------------|

AKC

D1



US005173429A

# United States Patent [19]

Gaddy et al.

[11] Patent Number: 5,173,429

[45] Date of Patent: Dec. 22, 1992

- [54] *CLOSTRIDIUM LJUNGDAHLII*, AN ANAEROBIC ETHANOL AND ACETATE PRODUCING MICROORGANISM
- [75] Inventors: James L. Gaddy; Edgar C. Clausen, both of Fayetteville, Ark.
- [73] Assignee: The Board of Trustees of the University of Arkansas, Little Rock, Ark.
- [21] Appl. No.: 612,221
- [22] Filed: Nov. 9, 1990
- [51] Int. Cl.<sup>5</sup> ..... C12N 1/20; C12P 7/06; C12N 1/00
- [52] U.S. Cl. .... 435/252.7; 435/161; 435/163; 435/135; 435/842
- [58] Field of Search ..... 435/281, 282, 252.7, 435/163, 161, 170, 842

## [56] References Cited

## U.S. PATENT DOCUMENTS

4,652,526 3/1987 Hsu

## OTHER PUBLICATIONS

Tanner and Gaddy, Amer. Soc. for Microbiology Annual Mtg. 1990 Abstract #R-21, p. 249.

J. Bacteriology, Sep. 1979, Wiegel et al., pp. 800-810, vol. 139, No. 3.

Advanced Studies of Biological Indirect Liquefaction of Coal: Topical Report on Task I: Cultural Identification, Jan. 1989, U.S. Dept. of Energy, Pittsburgh, En-

ergy Technology Center, Contract No. DE-AC2-2-88PC79813.

Vega et al., The Biological Production of Ethanol From Synthesis Gas, Applied Biochemistry and Biotechnology, vol. 20/21, 1989, pp. 781-797.

Barik et al., Biological Production of Alcohols from Coal Through Indirect Liquefaction, The Humana Press, 1988, pp. 363-378.

Klasson et al., Biological Production of Liquid and Gaseous Fuels From Synthesis Gas, Applied Biochemistry and Biotechnology, vol. 24/25, 1990, pp. 1-20.

Lundback et al., Parameters Affecting the Kinetics of Ethanol Production from CO, CO<sub>2</sub>, and H<sub>2</sub> by *Clostridium ljungdahlii*, 12th Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, Tenn.

Primary Examiner—Douglas W. Robinson

Assistant Examiner—Jane A. Williams

Attorney, Agent, or Firm—Hermann Ivester

## [57]

## ABSTRACT

A newly discovered microorganism was isolated in a biologically pure culture and designated *Clostridium ljungdahlii*, having the identifying characteristics of ATCC No. 49587. Cultured in an aqueous nutrient medium under anaerobic conditions, this microorganism is capable of producing ethanol and acetate from CO and H<sub>2</sub>O and/or CO<sub>2</sub> and H<sub>2</sub> in synthesis gas. Under optimal growth conditions, the microorganism produces acetate in preference to ethanol. Conversely, under non-growth conditions, ethanol production is favored over acetate.

7 Claims, 2 Drawing Sheets

5,173,429

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# CLOSTRIDIUM LJUNGDAHLII, AN ANAEROBIC ETHANOL AND ACETATE PRODUCING MICROORGANISM

This invention was made with Government support under Contract No. De-AC22-85PC80012 awarded by the Department of Energy.

## BACKGROUND OF THE INVENTION

The present invention relates to a biologically pure culture of an anaerobic microorganism and its isolation. More specifically, the present invention relates to the production of ethanol and acetate from carbon monoxide, carbon dioxide, and hydrogen, the major components of synthesis gas.

As a major source of both fuel and chemicals, coal represents the United States' largest fossil energy source. Conversion of coal to a more efficient energy source and a valuable chemical feedstock has been achieved by gasification processes. In most gasification processes, the coal is hydrogasified by adding steam, and energy (20-70 atm), with temperatures reaching as high as 2730° C. Gasification of solid fuels like coal produces synthesis gas, a gas typically consisting of more than 50 percent hydrogen and carbon monoxide. In addition, carbon dioxide is also produced along with small amounts of methane and sulfur gases. Suitable for further processing, synthesis gas can be used as a major intermediate in the production of liquid fuels such as alcohols and petrochemicals.

Recent efforts have focused on developing more efficient and economical methods for using available energy resources. To this end, considerable interest has developed in the biological conversion of synthesis gas components to liquid fuels in view of the distinct advantages offered by microbial processes over traditional catalytic processes.

Chemical conversions require high temperatures and pressures, resulting in losses in thermal efficiency and high energy costs. On the other hand, microbial conversions occur at ambient temperatures and pressures, resulting in substantial energy and equipment savings. Also, product yields from microbial conversions are quite high as compared to chemical conversions, since the microorganism utilizes only a small fraction of the substrate for energy and growth. Further, under proper conditions, microbial conversions are quite specific, generally converting a substrate into a single product. It follows that these conversions would have useful application in industrial processes such as chemical feedstock and fuel production.

Conversion of coal to liquid fuels by microorganisms occurs by either direct or indirect biological action. Although direct biological action on coal has tremendous potential, there are several disadvantages exhibited by the processes such as the apparent toxicity of liquefied coal products and waste water streams that result from the coal conversion process.

A more promising biological approach is the indirect coal conversion of synthesis gas by microorganisms capable of producing alcohols and acids from CO, CO<sub>2</sub>, and H<sub>2</sub>. A two-step process is required. First, synthesis gas is produced by catalytic action on coal using conventional gasification techniques. The biological conversion of synthesis gas to liquid fuels involves contacting the gas and microorganisms in liquid culture. The gas is then absorbed at the gas-liquid interface and dif-

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fuses through the culture medium to the cell surface to be consumed by the microorganisms.

Several species of clostridia have been found to utilize CO and CO<sub>2</sub>/H<sub>2</sub> as substrates. *Clostridium thermoaceticum* utilizes CO to produce acetate. Similarly, *Clostridium thermoautotrophicum* produces acetate and butyrate from CO, CO<sub>2</sub> and H<sub>2</sub>. Other clostridial species have been shown to ferment CO<sub>2</sub> and H<sub>2</sub> to formate, acetate, and butyrate. However, no microorganism has, heretofore, been known to form ethanol from synthesis gas components.

## SUMMARY OF THE INVENTION

The present invention is directed to the isolation of a biologically pure culture of a newly discovered anaerobic microorganism, *Clostridium ljungdahlii*. The present invention is further directed to the unique capability of the organism to produce ethanol and acetate from synthesis gas.

In an embodiment of the present invention, the organism produces acetate in preference to ethanol under normal growth conditions, typically between pH 5.0 and 7.0.

In a further embodiment of the present invention, ethanol production is favored over acetate production at pH between 4.0 and 4.5.

In addition, the present invention includes morphological and unique fermentation characteristics that support the scientific conclusion that this microorganism is distinct from previously reported microbiological isolates. Accordingly, an unrestricted deposit of said microorganism was made with the American Type Culture Collection (ATCC), Rockville, Md. on Aug. 17, 1990, under Accession No. 49587.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the product concentrations (g/L) of ethanol and acetate attained in liquid batch cultures with a continuous gas feed with *Clostridium ljungdahlii*. Plot A represents cell concentration (mg/L) versus time. Plot B represents ethanol concentration (g/L) versus time. Plot C represents acetate concentration versus time.

FIG. 2 illustrates ethanol concentrations (g/L) attained in a two-stage CSTR system with *Clostridium ljungdahlii* in the presence of yeast extract and without yeast extract. Plot A represents the first reaction mixture. Plot B represents the second reactor.

FIG. 3 illustrates the molar product ratio of ethanol/acetate attained in a two-stage CSTR system with *Clostridium ljungdahlii* in the presence of yeast extract and without yeast extract. Plot A refers to the first reactor. Plot B refers to the second reactor.

## DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

The present invention describes the isolation of a biologically pure culture of the new anaerobic microorganism, *Clostridium ljungdahlii*, sometimes referred to hereinafter as "*C. ljungdahlii*". Once isolated, biochemical and morphological properties of the organism were analyzed. Although similar to other organisms in its ability to ferment certain carbohydrates, *C. ljungdahlii* is distinguished in its ability to produce ethanol from CO and H<sub>2</sub>O and/or CO<sub>2</sub> and H<sub>2</sub>, the major components of synthesis gas. The organism also produces acetate from synthesis gas. In view of its ability to produce ethanol, as discussed in detail below, this organism

is deemed a new species designated *Clostridium ljungdahlii*, ATCC No. 49587 being representative of this species.

The microorganism of the present invention was initially isolated from chicken waste. The isolation of *C. ljungdahlii* in a biologically pure form was accomplished using the anaerobic techniques according to Hungate (Bacteriol. Rev. 14: 1-49, 1950), Bryant (Am. J. Clin. Nutr. 25: 1324-1328, 1972), and Balch and Wolfe (Appl. Environ. Microbio. 32: 781-791, 1976), and the methodology of such technique is hereby incorporated in the disclosure by reference.

The medium used for isolation and culture identification studies of the new strain, in accordance with this invention, has the preferred composition as shown in Table 1.

TABLE 1

| Isolation Medium             | Per 100 mL of Medium |
|------------------------------|----------------------|
| Pfennig's minerals           | 5.0 mL               |
| Pfennig's trace minerals     | 0.1 mL               |
| B-vitamins                   | 0.1 mL               |
| Yeast extract                | 0.1 g                |
| Resazurin                    | 0.5 mL               |
| Distilled or deionized water | 100 mL               |

Once isolated, the biologically pure culture was maintained in isolation medium (see Table 1) and synthesis gas (65% CO, 24% H<sub>2</sub>, and 11% CO<sub>2</sub>). The medium was maintained at a pH of 5.0 and a temperature of 37° C. The culture was stored in a non-shaking incubator (Precision Scientific), and transferred every two weeks.

Extensive studies were subsequently performed to characterize the biochemical properties of the organism. To this end, batch fermentations were initially conducted, as described below, utilizing a variety of substrates.

Media preparation (see Table 1) was carried out in an atmosphere of 80% N<sub>2</sub> and 20% CO<sub>2</sub>, as described by Hungate (Meth. Microb. 3B: 117, 1969) and Lungdahl and Wiegel (Manual of Industrial Microbiology and Biotechnology, American Society for microbiology, pp. 84-96, 1986). The media was anaerobically transferred into serum bottles, sealed, and then autoclaved at 121° C. for 20 minutes. Reducing solutions (cysteine-HCl and sodium sulfide) were added to the serum bottles prior to inoculation with a seed culture. Different substrates were added to the medium. The bottles were placed in a shaker incubator at 37° C. during the experiments. Fermentation products were measured in both the liquid and gas samples that were removed from the serum bottles. Analyses of the alcohol and acid content of the fermentation products were performed by gas chromatography.

To monitor cell viability in each culture, cell concentrations were determined by comparing optical density readings with a standard calibration curve at 580 nm in a Spectronic-20 or Spectronic-21 spectrophotometer (Bausch and Lomb).

Batch fermentations revealed that *C. ljungdahlii* is capable of utilizing various carbon and energy sources (as illustrated in Example 5 and Table 5). Under typical growth conditions (pH 5.0-7.0), it was found that the organism produces significant levels of acetate as compared to ethanol levels when utilizing synthesis gas as a substrate. Preliminary studies show that the organism

also produces acetate from a variety of sugar substrates and other carbon sources.

As will be shown in the following examples, a product molar ratio (moles ethanol/moles acetate) of 0.05 and an ethanol concentration of less than 1 g/L was achieved with batch fermentations using a synthesis gas substrate at pH 5.0. By continually optimizing the culture, efforts to improve the product molar ratio in favor of ethanol production were successful. Specifically, it has been found that ethanol production is favored over acetate production under non-growth conditions. For example, by altering the pH and nutrient levels of the organism, ethanol concentrations of approximately 7 g/L and a product ratio of 7 grams ethanol/gram of acetate (9 mols ethanol/mol acetate) were obtained. Improvements were further obtained by utilizing a variety of techniques, including a two-stage continuous contacting (CSTR) system, pH shift, and alternative medium constituents. In a two-stage CSTR, acetate production has been eliminated in the second stage.

By way of example, and not limitation, the following examples serve to further illustrate the present invention in its preferred embodiments.

## EXAMPLE 1

Isolation of *Clostridium ljungdahlii*

Samples from chicken waste were collected under anaerobic conditions. In serum bottles, one ml of sample was added anaerobically to 9 ml of nutrient medium described in Table 1. 0.2 ml of 2.5% NaS<sub>2</sub> was added for every 10 ml of solution in order to eliminate any dissolved oxygen. 0.1 ml of either BESA or monensin was added for every 10 ml of solution to block methane production and facilitate alcohol and acid production. It should be noted that all inoculation procedures were performed anaerobically. Further, the medium contained CO (64%, 2 atm) in CO<sub>2</sub> and N<sub>2</sub> or synthetic synthesis gas (73% CO, 10% CO<sub>2</sub>, 15% H<sub>2</sub>, and 2% CH<sub>4</sub>) as the primary carbon source, although variations in the composition of these gases is acceptable for experimental purposes.

The samples were incubated in a shaking incubator with the pH between 5.0 and 7.0. Once gas consumption had begun, 1.0 ml of the inoculated medium was transferred to new medium. The samples required between 1 and 6 months, but typically 3 months, to initiate gas consumption. The original sample was not discarded. If no gas consumption occurred in the original sample after a period of a month to a month and a half, the sample was transferred to new medium, thus ensuring that the nutrients required for cell growth would not be depleted.

Next, several dilutions were performed in order to purify the culture. The culture was diluted up to 10<sup>10</sup> times with the nutrient medium and then incubated at 37° C. Dilutions of this magnitude helped to ensure the isolation of the most predominant organism. Bacterial growth occurred in all culture tubes up to a dilution of 10<sup>8</sup> and in some tubes at 10<sup>9</sup> and 10<sup>10</sup> dilution. Typically, aliquots from the third dilution were spread onto agar plates. However, this step would vary depending upon the density of the cell culture. The plates were then incubated anaerobically at 37° C. The nutrient medium, supplemented with 2% agar, was used for the agar plates. Colonies were isolated from the plates and then inoculated into fresh medium for use in experiments.

concentrations produced in Reactor A from those exiting Reactor B.

Results indicate that the pH shift between the reactors in this system was a significant step in causing *Clostridium ljungdahlii* to shift from acetate production or growth phase to producing ethanol. Further, it appeared that the removal of nutrient sources promoted ethanol production at the expense of acetate production. The substitution of cellobiose indicates that removal of essential nutrients required by the organism to grow results in a shift by the organism from acetate production and a growth phase to ethanol production.

### EXAMPLE 5

#### Fermentations Using Diverse Substrates

Table 5 lists those substrates tested for growth as the sole carbon and energy source for the organism. 1 g/L of yeast extract was added to the medium in addition to the substrate, and all fermentations were conducted at an initial pH of 6.0. As noted in the table, growth occurred when CO, CO<sub>2</sub>/H<sub>2</sub>, ethanol, pyruvate, xylose, arabinose, and fructose were used as substrates. *Clostridium ljungdahlii* grew weakly in the presence of glucose, fumarate, ribose, and casamino acids.

TABLE 5

| Growth of <i>Clostridium ljungdahlii</i> on Substrates as the Sole Carbon/Energy Source |   |           |   |
|-----------------------------------------------------------------------------------------|---|-----------|---|
| H <sub>2</sub> : CO <sub>2</sub>                                                        | + | ribose    | w |
| CO                                                                                      | + | xylose    | + |
| formate                                                                                 | - | arabinose | + |
| methanol                                                                                | - | fructose  | + |
| ethanol                                                                                 | + | glucose   | w |
| pyruvate                                                                                | + | galactose | - |
| lactate                                                                                 | - | mannose   | - |
| glycerol                                                                                | - | sorbitol  | - |
| succinate                                                                               | - | sucrose   | - |
| fumarate                                                                                | w | maltose   | - |
| citrate                                                                                 | - | lactose   | - |
|                                                                                         |   | starch    | - |

TABLE 5-continued

| Growth of <i>Clostridium ljungdahlii</i> on Substrates as the Sole Carbon/Energy Source |   |
|-----------------------------------------------------------------------------------------|---|
| casamino acids                                                                          | w |
| + positive growth                                                                       |   |
| - no growth                                                                             |   |
| w weak growth                                                                           |   |

It should be understood that the present invention involves the various embodiments associated with the discovery of *Clostridium ljungdahlii* and its use in all respects, and is not to be construed as limited to any specific aspect or embodiment except as defined by the lawful scope of the appended claims.

We claim:

1. A biologically pure culture of the microorganism *Clostridium ljungdahlii*, having all of the identifying characteristics of ATCC No. 49587.
2. A biologically pure culture of the microorganism *Clostridium ljungdahlii*, having the identifying characteristics of ATCC No. 49587, said culture, under anaerobic conditions, having the ability to produce ethanol and acetate upon fermentation in an aqueous nutrient medium containing synthesis gas as a substrate.
3. The biologically pure culture of claim 2, wherein said substrate is selected from the group consisting essentially of CO, CO<sub>2</sub>, and H<sub>2</sub>.
4. A biologically pure culture of the microorganism *Clostridium ljungdahlii*, having all of the identifying characteristics of ATCC No. 49587, said culture, under anaerobic conditions, having the ability to produce acetate in an aqueous nutrient medium comprising sources of carbon.
5. The biologically pure culture of claim 4, wherein said carbon source is a sugar.
6. A biologically pure culture of the microorganism *Clostridium ljungdahlii*, having all of the identifying characteristics of ATCC No. 49587, said culture exhibiting the following characteristics: rod-shaped, motile, gram-positive, and strictly anaerobic.
7. A biologically pure culture of the microorganism *Clostridium ljungdahlii*, having all of the identifying characteristics of ATCC No. 49587, said culture, under anaerobic conditions, having the ability to utilize pyruvate, xylose, arabinose, and fructose as substrates for growth.

\* \* \* \* \*

## United States Patent [19]

Lawford

[11] Patent Number: 4,812,410

[45] Date of Patent: Mar. 14, 1989

## [54] CONTINUOUS PROCESS FOR ETHANOL PRODUCTION BY BACTERIAL FERMENTATION

[75] Inventor: Hugh G. Lawford, Mississauga, Canada

[73] Assignee: George Weston Limited, Toronto, Canada

[21] Appl. No.: 735,913

[22] Filed: May 20, 1985

## Related U.S. Application Data

[63] Continuation of Ser. No. 722,492, Apr. 12, 1985.

[51] Int. Cl.<sup>4</sup> ..... C12P 7/06; C12N 1/20; C12N 1/38

[52] U.S. Cl. .... 435/161; 435/244; 435/813

[58] Field of Search ..... 435/161, 244, 253, 822, 435/813

## [56] References Cited

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|           |         |                    |         |
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| 4,127,447 | 4/1978  | Griffith et al.    | 435/161 |
| 4,292,406 | 9/1981  | Ljungdahl et al.   | 435/42  |
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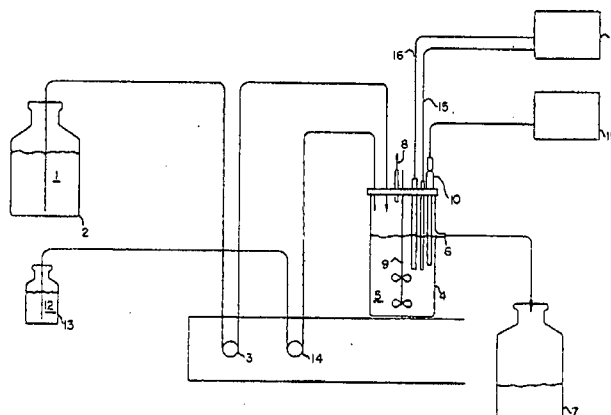
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## [57] ABSTRACT

A continuous process for the production of ethanol by fermentation with strains of *Zymomonas* is provided. Metabolic processes are limited by the nutrients nitrogen, potassium and phosphorus. When growth is limited by one of these nutrients, the biomass expresses its maximum value for both  $q_s$  and  $q_p$  at any given value of  $D$  and  $S_p$ . The process is conducted at a lower biomass concentration and a higher specific rate of ethanol formation than a similar process conducted with a nutrient medium that is not limited in nitrogen, potassium or phosphorus. A method of improving performance of *Zymomonas* in continuous ethanol fermentation at increased temperatures is also provided.

18 Claims, 1 Drawing Sheet



4,812,410

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amount of potassium dihydrogen phosphate employed and the results obtained are reported in Table 6.

TABLE 6

| Amount of Assimilable Phosphorus, as $\text{KH}_2\text{PO}_4$ , Required to Achieve Maximal Rate of Sugar Utilization and Ethanol Production by <i>Z. mobilis</i> ATCC 29191 in Continuous Culture at Fixed Dilution Rate ( $0.15 \text{ hr}^{-1}$ ) as a Function of Feed Sugar Concentration |            |                  |       |       |                      |       |       |                                 |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|------------------|-------|-------|----------------------|-------|-------|---------------------------------|
| $D = 0.15 \text{ hr}^{-1}$                                                                                                                                                                                                                                                                     |            |                  |       |       |                      |       |       |                                 |
| $S_p$<br>g/L                                                                                                                                                                                                                                                                                   | Eth<br>g/L | Excess Phosphate |       |       | Phosphate Limitation |       |       | $\text{KH}_2\text{PO}_4$<br>g/L |
|                                                                                                                                                                                                                                                                                                |            | X<br>g/L         | $q_s$ | $q_p$ | X<br>g/L             | $q_s$ | $q_p$ |                                 |
| 20                                                                                                                                                                                                                                                                                             | 9.6        | 0.58             | 5.2   | 2.5   | 0.42                 | 7.2   | 3.4   | 0.04                            |
| 60                                                                                                                                                                                                                                                                                             | 28         | 1.73             | 5.2   | 2.5   | 1.25                 | 7.2   | 3.4   | 0.13                            |
| 110                                                                                                                                                                                                                                                                                            | 52         | 3.17             | 5.2   | 2.5   | 2.29                 | 7.2   | 3.4   | 0.23                            |

Units:  $q_s = \text{g glu/g cell-hr}^{-1}$ ;  $q_p = \text{g eth/g cell-hr}^{-1}$ .

The data in Table 6 show that the results obtained with phosphorus-limited fermentation are analogous to the results obtained with nitrogen-limited and potassium-limited fermentations. Phosphorus-limited fermentation can be carried out at lower biomass concentration, higher specific rate of glucose uptake, higher specific rate of product formation and comparable product yield as compared to fermentation carried out under conditions of phosphorus-excess. The data show that the amount of phosphorus required for phosphorus-limited fermentation is proportional to the concentration of glucose in the feed stream.

When this invention is carried out with phosphorus-limited fermentation and when it is necessary to control the pH of the fermentation medium, a pH regulating agent or buffering agent other than a phosphorus-containing compound should be employed. The additional source of phosphorus from a phosphorus-containing pH regulating agent may prevent the degree of control of nutrient-limitation required by the invention. For phosphorus-limited fermentation  $\text{KH}_2\text{PO}_4$  can be employed as a buffer and NaOH titrant diluted to 0.5N in order to avoid large changes in pH during automatic titration.

#### FORMULATING THE NUTRIENT MEDIUM

The identity of the chemical constituents in the nutrient medium and the amount of each constituent should be sufficient to meet the elemental requirements for cell mass and ethanol production and should supply appropriate energy for synthesis and maintenance. The nutrient medium should contain carbon, nitrogen, potassium, phosphorus, sulfur, magnesium, calcium and iron in required amounts. The chemical constituents should also meet specific nutrient requirements including vitamins and trace minerals.

As the assimilable source of nitrogen, various kinds of inorganic or organic salts or compounds can be included in the nutrient medium. For example, ammonium salts, such as ammonium chloride or ammonium sulfate, or natural substances containing nitrogen, such as yeast extract, peptone, casein hydrolysate or corn steep liquor, or amino acids, such as glutamic acid, can be employed. These substances can be employed either singly or in combination of two or more.

Examples of inorganic compounds that can be included in the culture medium are magnesium sulfate, potassium monohydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, magnesium sulfate, calcium chloride, iron chloride, magnesium chloride, zinc sulfate, cobalt chloride, copper chloride, borates and molybdates.

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Organic compounds that may be desirable in the fermentation include, for example, vitamins, such as biotin, calcium pantothenate, and the like, or organic acids, such as citric acid, or amino acids, such as glutamic acid. It has been found, however, that biotin is not required in the growth medium.

Fermentation aids that are non-toxic to the microorganism can be included in the nutrient medium and fermentation broth. For example, an anti-foaming agent in a minor amount has been found to be advantageous.

Examples of nutrient media that have been found suitable for use in this invention are described in Table 7.

TABLE 7

| Chemical Composition of Growth Media for Continuous Culture of <i>Z. mobilis</i> |                             |                            |
|----------------------------------------------------------------------------------|-----------------------------|----------------------------|
| INGREDIENT                                                                       | SEMI-SYNTHETIC MEDIUM (g/L) | DEFINED SALTS MEDIUM (g/L) |
|                                                                                  |                             |                            |
| D-Glucose (anhydrous) approx.                                                    | 100                         | 100                        |
| Yeast Extract (Difco)                                                            | 5                           | —                          |
| $\text{NH}_4\text{Cl}$                                                           | 2.4                         | 2.4                        |
| $\text{KH}_2\text{PO}_4$                                                         | 3.48                        | 3.48                       |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$                                        | 1.0                         | 1.0                        |
| $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$                                        | 0.01                        | 0.01                       |
| Citric Acid                                                                      | 0.21                        | 0.21                       |
| Vitamins                                                                         |                             |                            |
| Ca-pantothenate                                                                  | 0.001                       | 0.001                      |
| Biotin                                                                           | 0.001                       | 0.001                      |
| Antifoam as required                                                             |                             |                            |

The semi-synthetic medium is suitable for use in the nitrogen-limited fermentation according to this invention. It will be understood that the composition of this medium will depend on the technical quality of the nitrogen source. For example, yeast extract from a batch from one commercial source may exhibit a different potency with respect to the content of assimilable nitrogen than a yeast extract from a different batch or from another commercial source. Also, the amount of the nitrogen source required in the medium will depend on the degree of hydration; anhydrous chemicals are preferred and were employed in the Examples and in the fermentations reported in the Tables.

The defined salts medium is suitable for use in carrying out the potassium-limited and phosphorus-limited fermentations. Only inorganic sources of nitrogen, such as ammonium salts, are employed in defined salts media. In the experiments reported in the foregoing Tables and Examples, the defined salts medium was used in the potassium- and phosphorus-limited fermentations, with the following exceptions.

Ordinarily, the phosphorus in the nutrient medium is supplied as a salt having an anion that is substantially non-toxic to the microorganism and that does not substantially inhibit normal metabolic processes. While a potassium salt, such as potassium dihydrogen phosphate, is typically employed for nitrogen-limited and phosphorus-limited fermentations, a sodium salt is preferably substituted for the potassium salt in potassium-limited fermentation. For example, sodium dihydrogen phosphate instead of potassium dihydrogen phosphate can be utilized. Since the amount of available potassium must be precisely known under potassium-limited conditions, this substitution makes it easier to control the relative proportions of nutrients.