

Clarke, Modet & C^o

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
 DIVISION DE NUEVAS CREACIONES
 E. S. D.

Re: Expediente No. 03-002.550

Solicitud de patente a nombre de **Emmaus Foundation, Inc**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución adjunta, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **Emmaus Foundation, Inc**, domiciliada en Fayetteville, Arkansas, Estados Unidos de América, solicitante de la patente de la referencia, en respuesta al oficio No. 16120 notificado por fijación en lista el 19 de diciembre de 2008 relacionado con el concepto técnico de fondo No. 3517, me permito manifestar:

1. Nivel inventivo.

Como primera medida nos permitimos recordar lo establecido por la Decisión 486 en cuanto a este requisito en el artículo 18: *“Una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”* (El subrayado es nuestro).

En este sentido, es necesario señalar que el señor examinador no realizó un examen concienzudo de la materia objeto de la presente invención, toda vez que la misma no resulta evidente o susceptible de derivación obvia del estado de la técnica como más abajo se demostrará.

En efecto, el examinador en su concepto técnico N0. 3517 que:

“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 ijungdablii; 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 H₂O y/o CO₂ y H₂(columna 2, línea 62 a 66).

D2 divulga un proceso continuo para la producción de etanol por fermentación con cepas de *Zymomonas*, 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. Portante, ante la información divulgada en 1 y D2 la invención carece de nivel inventivo.”

Sobre este particular, es menester señalar que el documento D1 no hace ninguna sugerencia al hecho que el etanol pueda ser obtenido de manera preferida a pH entre 4.5 y 5.5 a través de la bacteria *C. ljungdahlii*. Es más, D1 sugiere que a estos

valores de pH y a condiciones normales de crecimiento el microorganismo C. ljungdahlii tiende más a la producción de acetato que de etanol.

En este sentido, atentamente dirigimos la atención del señor examinador a la columna 2 entre las líneas 20 a 23 de D1 donde se expone que *"En una modalidad de la presente invención, el microorganismo produce acetato en lugar de etanol bajo condiciones normales de crecimiento, típicamente entre pH 5,0 y 7,0."* (la traducción es nuestra).

Ahora bien, no existe razón alguna para que una persona versada en la materia combinara las anteriores enseñanzas con lo mostrado en el documento D2. En primer lugar, porque pensaría que la producción de etanol mediante la bacteria C. ljungdahlii estaba supeditada a la manipulación del medio de cultivo, principalmente en el extracto de levadura y segundo, debido a la naturaleza de la bacteria revelada en la anterioridad D2, toda vez que las Zymomonas son bacterias gram negativas, es decir, bacterias con paredes celulares más delgadas y finas en comparación con la bacteria C. ljungdahlii que pertenece a la clase de las gram positivas, lo cual forzosamente conduciría a la persona versada a pensar que los medios de cultivo de la zymomona no le sirven a la C. ljungdahlii, debido a la capacidad de absorción de los nutrientes por parte de las membranas celulares.

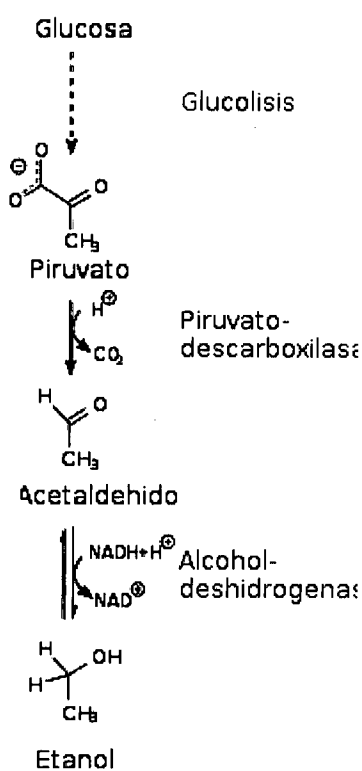
Aún más, al revisar las enseñanzas del documento D2, se puede corroborar que no existe dentro de la descripción de dicho antecedente sugerencia alguna en cuanto a la utilidad del pantotenato de calcio en medios de cultivo para otra clase de bacterias y en especial para gram positivas como la C. ljungdahlii. De hecho, D2 en la columna 2 entre las líneas 32 a 40 señala que para mejorar el desempeño de la fermentación de cepas de Zymomonas, los procesos metabólicos deben ser limitados por medio de nutrientes como nitrógeno, potasio o fósforo. Sin embargo, no hace ninguna sugerencia a que el pantotenato de calcio tenga alguna relevancia dentro de caldos de cultivo de C. ljungdahlii.

Sobre este particular, dirigimos la atención del señor examinador a la columna 14, entre las líneas 1 a 6 de D2, donde simplemente se menciona que *"los compuestos orgánicos que podrían ser deseables en la fermentación incluyen, por ejemplo, vitaminas, tal como biotina, pantothénato de calcio, y similares o ácidos orgánicos tal como ácido cítrico, o aminoácidos tal como ácido glutámico. Se ha encontrado sin embargo que la biotina no es requerida en el medio de crecimiento."*

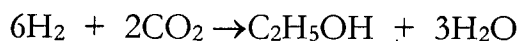
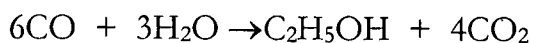
Así las cosas, una persona medianamente versada en la material técnica correspondiente no hubiera encontrado conexión alguna entre lo revelado en D2 y lo enseñado en D1 para llegar de manera evidente al método reclamado en la solicitud en estudio, toda vez que D1 sugiere que el etanol se produce a partir de una modificación en el extracto de levadura en el medio de cultivo a pH 5.0 y D2 enseña medios de cultivo para bacterias zymomonas, que son gram negativas y que producen etanol a partir de la degradación de glucosa, lo cual es

absolutamente diferente de lo planteado en la solicitud en estudio, donde el alcohol se produce a partir de CO_2 y por una ruta metabólica diferente a la que se sigue cuando se degrada glucosa.

En ese orden de ideas, atentamente nos permitimos hacer énfasis en el hecho que las zymomonas son bacterias que requieren de azúcares, como la glucosa, como fuente de carbono. Adicionalmente, estas bacterias al encontrarse en condiciones anaeróbicas a través de la ruta de la glicólisis transforman glucosa en piruvato y éste a su vez en etanol, como se muestra en el siguiente esquema:



En contraste con lo anterior, la ruta de síntesis realizada por cepas de la bacteria acetogénica y anaeróbica *Clostridium ljungdahlii* a partir de óxidos de carbono, llevan a la producción de ácido acético como producto principal y etanol mediante la conversión tanto del monóxido como del dióxido de carbono, como se muestra en las siguientes ecuaciones estequiométricas generales:



En consecuencia, la vía de síntesis mediante la cual se obtiene etanol a partir de zymomonas comprende la participación de glucosa y pantotenato para el crecimiento de las bacterias en este medio, en tanto que el efecto que el pantotenato realiza sobre el *Clostridium ljungdahlii* es incrementar la producción de etanol. Por lo tanto, esta es una razón más para evidenciar el nivel inventivo de la solicitud en estudio, toda vez que no habría sido evidente para una persona medianamente versada en la materia incorporar pantotenato a un sistema de cultivo que emplea un sustrato gaseoso para llevar a cabo la síntesis selectiva de etanol en bacterias de *Clostridium ljungdahlii*, las cuales lo realizan por una vía de síntesis totalmente diferente a la seguida por las zymomonas.

En este sentido, respetuosamente nos permitimos señalar que no basta únicamente con afirmar que D1 revele el método de producción de etanol con C ljungdahlii y que una persona normalmente versada en la técnica buscaría una anterioridad como D2 que revela medios de cultivo que pueden tener pantotenato de calcio, sin demostrar de manera contundente cómo a dicha persona se le hubiera ocurrido realizar tal combinación de anterioridades sin siquiera encontrar alguna sugerencia en D1 y D2 en relación con posibles beneficios para un método de producción de etanol donde la cepa C ljungdahlii sea cultivada con pantotenato de calcio.

Ahora bien, el análisis de nivel inventivo realizado por el señor examinador es claramente el resultado de un análisis del tipo “hindsight”. De hecho, este tipo de análisis no es permitido para desvirtuar el concepto de altura inventiva de una invención.

En este tipo de estudio, como se puede constatar en este caso, el examinador no ha enfocado su análisis en el momento en el que el solicitante plantea un problema con el fin de solucionar fallas técnicas existentes en los productos convencionales de un campo técnico específico, sino que se encauza en la solución dada al problema planteado y en la búsqueda de documentos con conceptos análogos.

A este respecto dirigimos la atención del señor examinador al “Manual para Examen de Solicitudes de Patentes de Invención en las Oficinas de Propiedad Industrial de los Países de la Comunidad Andina, publicado en la página Web de la Comunidad Andina, donde se establece en la página 74 en cuanto al nivel inventivo “...que la búsqueda de anterioridades se efectúa a posteriori, tomando como punto de partida la misma invención,...el examinador debe realizar el esfuerzo intelectual de colocarse en la situación que ha tenido que afrontar el técnico con conocimientos medios en la materia en un momento en que la invención no era conocida, es decir antes de la invención”.(el resaltado es nuestro)

En el presente caso, para el examinador puede resultar “obvio” que el objeto de la presente solicitud se derive de la materia objeto de los documentos citados como referencia D1 y D2 conociendo la acción del pantotenato de calcio a partir de

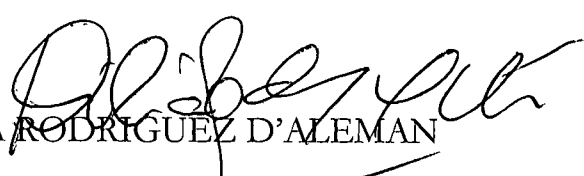
la solicitud en estudio, sin dar mayor importancia al trabajo desplegado y a la capacidad inventiva del solicitante para lograr llegar a la solución del problema planteado. Es decir, que el examinador no hace su análisis partiendo del problema mismo que existía en la técnica en el momento en que el solicitante dirige todos sus esfuerzos para encontrar una solución, sino que enfoca su estudio en el momento en que el problema ya fue resuelto.

En consecuencia, para una persona normalmente versada en la materia técnica, como se establece en el artículo 18 de la Decisión 486, no habría sido obvia la combinación de un documento como D1 que hace alusión al aislamiento de microorganismos del tipo C. ljungdahlii susceptibles de producir tanto acetato como etanol, con las enseñanzas de la referencia D2 que sugieren la presencia de compuestos orgánicos como el pantotenato de calcio en medios de cultivo para cepas totalmente diferentes y que no pertenecen siquiera al mismo género del clostridium.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: “*habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares*” y “*(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso*”, respectivamente (El subrayado es nuestro).

En consecuencia, pedimos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,


DILIA MARIA RODRIGUEZ D'ALEMAN
CC. 41.654.882
TP 20824 CSJ

Clarke, Modet & C^o

COLOMBIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

REF: **EXPEDIENTE No. 03.002.550**
SOLICITUD DE PATENTE A NOMBRE DE MEAUX
FOUNDATION, INC.

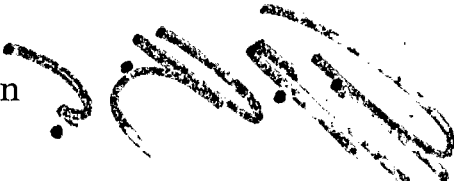
Yo, JIMENA ESCOBAR URIBE, identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53 Piso 6, atentamente digo a usted que sustituyo el poder con que obro a la doctora DILIA RODRIGUEZ D'ALEMAN, domiciliada en Bogotá, D.C., portadora de la tarjeta profesional No. 20.824, con las mismas facultades con que me fueron conferidas para este caso.


JIMENA ESCOBAR URIBE

C.C. 39.779.452 de Usaquén

T.P. 68.228

Acepto sustitución


DILIA RODRIGUEZ D'ALEMAN

C.C. 41.654.882 de Bogotá

T.P. 20.824

PRESENTACION PERSONAL

El anterior escrito fue presentado ante el
NOTARIO ONCE DEL CIRCULO DE BOGOTA, D.C.
personalmente por

DEYANIRA TOLOBAR

U2925

quien exhibió la c.c. *34779452* de *UDTOLIBEN*
y Tarjeta Profesional No. *68 228* C.S.J.

Fecha *1 ABR 2009*

Monsieur

Guillermo Chávez Cárdenas



[Signature]
REPUBLICA DE COLOMBIA
Circulo de Bogotá D.C.
Circulo de Bogotá D.C.

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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 KH_2PO_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 hr^{-1}) as a Function of Feed Sugar Concentration									
$D = 0.15 \text{ hr}^{-1}$									
S_f g/L	Eth g/L	Excess Phosphate			Phosphate Limitation			KH_2PO_4	
		X g/L	q_s	q_p	X g/L	q_s	q_p	g/L	
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: $q_s = \text{g glu/g cell}\cdot\text{hr}^{-1}$; $q_p = \text{g eth/g cell}\cdot\text{hr}^{-1}$.

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 KH_2PO_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	—	
NH_4Cl	2.4	2.4	/
KH_2PO_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.

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CONTINUOUS PROCESS FOR ETHANOL PRODUCTION BY BACTERIAL FERMENTATION

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of application Ser. No. 722,492, filed Apr. 12, 1985, entitled CONTINUOUS PROCESS FOR ETHANOL PRODUCTION BY BACTERIAL FERMENTATION, the entire contents of which are relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

This invention relates to the bioconversion of a substrate by bacterial fermentation, and more particularly, to a continuous process for the production of ethanol by fermentation using strains of *Zymomonas* bacteria.

Bacterial ethanol fermentation has been known in the art for many years, and in recent years fermentation using strains of *Zymomonas mobilis* has received increasing attention. The *Z. mobilis* strains convert a suitable substrate, such as glucose or another sugar, to ethanol. Significantly higher specific rates of sugar uptake and ethanol production and improved yield compared to traditional yeast fermentation have been reported for these *Zymomonas* strains.

Fermentation by *Z. mobilis* has been carried out in batch and continuous culture. The fermentation product (ethanol) is dissolved in the liquid medium in the fermenter. The liquid medium is separated from solids (chiefly biomass) before the ethanol is recovered. Separation of these two phases early in the product-recovery process train is required. Before such a fermentation achieves commercial acceptance, however, productivity must be improved. The reported efforts to date have focused on developing more productive bacterial strains and modifying the configuration of the fermenter used in the fermentation. For example, improvement in ethanol productivity using a continuous culture with a cell recycle system has been reported with *Z. mobilis* strains.

Recovery of the fermentation product can be a complex and multifaceted task. A significant proportion of the overall cost in a fermentation plant often must be spent for ethanol recovery. A recently reported technique involves the use of a flocculent strain of *Z. mobilis* that settles in the fermenter allowing the supernatant containing the ethanol to be withdrawn while leaving a majority of the cells in the fermenter. This method is based on the well-known gravity sedimentation principle for separating liquids and solids. The more conventional approaches for separating fermentation broth from biomass involve the withdrawal of a portion of the culture medium from the fermenter and separation of the two phases by centrifugation or filtration techniques. Regardless of the technique employed, for a particular ethanol recovery process, it is desirable to reduce the quantity of biomass in order to reduce the load of solids on the sedimentation, centrifugation or filtration apparatus.

At the same time, however, the yield of ethanol from the fermentation must be maximized. Since product formation cannot occur in the absence of biomass, ethanol formation is dependent on cell mass concentration. In fact, the rate of ethanol production in the fermenter is directly proportional to the quantity of biomass in the fermenter under steady state conditions. Thus, within

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the limits of the metabolic regulatory controls of the microorganism and process dynamics, increasing the biomass in the fermenter while maintaining other conditions constant will shorten the time required to produce a given amount of ethanol. However, this seemingly simple approach for optimizing process performance will have an adverse effect on the ethanol recovery process because the load of solids on the separating equipment will be correspondingly increased.

It is well known that the substrate, such as glucose, is the largest item of raw material cost in the fermentation. Therefore, the presence of substrate in the effluent from the fermenter in a continuous fermentation is to be avoided. The continuous fermentation should be conducted at optimum process product yield, which occurs when the substrate is completely converted to ethanol and when the substrate is minimally diverted from product (ethanol) formation to cell mass synthesis (i.e., when the growth yield with respect to carbon substrate is minimized).

Thus, there exists a need in the art for a continuous process for the production of ethanol using strains of *Zymomonas* in which the substrate fed to the fermenter is converted to ethanol in as short a time as possible. The process should permit a reduction in the quantity of biomass in the fermenter in order to obtain a corresponding reduction in the load of solids on the ethanol recovery apparatus. In addition, the quantity of substrate in the effluent from the fermenter should be minimized.

SUMMARY OF THE INVENTION

This invention aids in fulfilling these needs in the art by providing a continuous process for the production of ethanol. The present invention relates to an improvement in the fermentation performance of strains of *Zymomonas* in continuous fermentations wherein metabolic processes are limited by various nutrients, these nutrients being nitrogen, potassium or phosphorus. When growth is limited by one of these nutrients, the biomass expresses its maximum value for both specific rate of substrate uptake (q_s) and specific rate of product formation (q_p) at any given value of dilution rate (D) and substrate concentration (S_f). One of the criteria for assessing the improvement in performance of *Zymomonas* according to this invention is the increase in the specific rate of ethanol (product) formation (q_p). The response of *Zymomonas* with respect to nutrient limitation by nitrogen, potassium or phosphorus could not be predicted from prior teachings. That *Zymomonas* expresses a maximal value for q_p (and q_s) under conditions of nutrient limitation was not a predictable phenomenon. The process of this invention 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 contains the nutrient in excess.

In one embodiment of this invention, a method of improving continuous ethanol production by bacterial fermentation with strains of *Zymomonas* is provided without changing the fermentation temperature. These results are achieved by carrying out the fermentation under nutrient-limiting conditions. The limiting nutrient is either nitrogen, potassium or phosphorus. The imposition of nutrient limitation makes it possible to conduct the fermentation at a lower biomass concentration at a given substrate concentration in the feed stream to the

Clostridium ljungdahlii sp. nov., an Acetogenic Species in Clostridial rRNA Homology Group I

RALPH S. TANNER,^{1*} LETRISA M. MILLER,¹ AND DECHENG YANG²

¹Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019, and
²Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Clostridium ljungdahlii sp. nov. strain ATCC 49587^T (T = type strain) was isolated from chicken yard waste for its ability to produce ethanol from synthesis gas. This gram-positive, motile, sporeforming rod's metabolism was primarily acetogenic. *C. ljungdahlii* grew with carbon monoxide, hydrogen and carbon dioxide, ethanol, pyruvate, arabinose, xylose, fructose, or glucose. Methanol, ferulic acid, lactate, galactose, and mannose did not support growth. The G+C content was 22 to 23 mol%. *C. ljungdahlii* is the first acetogen in clostridial 23S rRNA homology group I.

The potential for microbial production of liquid fuels (e.g., ethanol) and commodity chemicals (e.g., acetic acid) from synthesis gas (CO-H₂-CO₂) derived from coal has been recognized (27) and has prompted investigations of microorganisms which can metabolize the components of synthesis gas under anoxic conditions. A strain which could produce ethanol from synthesis gas was isolated from chicken yard waste (2, 25). In this report we describe this strain, a new, anaerobic, gram-positive, spore-forming rod which is the first acetogenic species in clostridial rRNA group I, as described by Johnson and Francis (15).

(A portion of this work has appeared previously [24].)

MATERIALS AND METHODS

The acetogenic clostridium described here was isolated by Sudhakar Barik at the University of Arkansas from an enrichment inoculated with chicken yard waste at an initial pH of 5.0 and incubated at 37°C under an atmosphere of synthesis gas (CO-H₂-CO₂-CH₄, 73:15:10:2) (2). This isolate was named *Clostridium ljungdahlii*. The single strain was designated strain PETC^T (T = type strain) and was deposited in the American Type Culture Collection as strain ATCC 49587^T.

Media. The media used for routine growth and characterization experiments were prepared by using strict anoxic techniques (1) and contained (per liter) 1.0 g of NH₄Cl, 0.8 g of NaCl, 0.1 g of KCl, 0.1 g of KH₂PO₄, 0.2 g of MgSO₄ · 7H₂O, 0.02 g of CaCl₂ · 2H₂O, 1.0 g of NaHCO₃, 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.2 g of cysteine hydrochloride, 0.2 g of Na₂S · 9H₂O, 10 ml of a trace metal solution (22), and 10 ml of a vitamin solution (22). An atmosphere containing N₂ and CO₂ (80:20, pressurized to 70 kPa) was used for growth with organic substrates, which were added to a final concentration of 5 g/liter. H₂-CO₂ (80:20, pressurized to 200 kPa) or CO-N₂-CO₂ (75:20:5, pressurized to 140 kPa) was used for growth on gaseous substrates. The initial medium pH was 5.8 to 5.9. Cultures were incubated at 37°C. The optimum pH (initial pH) was determined in medium containing fructose as the substrate and sodium acetate, 2-(N-morpholino)ethanesulfonic acid (MES), or N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Sigma Chemical Co., St. Louis,

Mo.) as a buffer (1.0 g/liter). The optimum temperature for growth was determined by using fructose as the substrate.

Fermentation balance. A fermentation balance was determined for growth on fructose or H₂-CO₂. Fructose was measured by using a phenol-sulfuric acid carbohydrate assay (9). Utilization of H₂-CO₂ (80:20) was determined by using a pressure transducer system (8) to measure the reduction of gas pressure in sealed culture tubes (1). Individual gases were measured with a gas chromatograph equipped with a thermal conductivity detector (Varian, Sugar Land, Tex.) and a Porapak Super Q column (Alltech, Deerfield, Ill.). The following equation was used to calculate the amount of gas consumed: 4H₂ + 2CO₂ → CH₃COOH + 2H₂O (19). Acetic acid was measured by ion-exclusion high-performance liquid chromatography on an Aminex HPX-87H column (Bio-Rad, Richmond, Calif.). Ethanol was measured enzymatically with alcohol dehydrogenase (kit 332-B; Sigma) or by gas chromatography on a GP 10% SP-1200/1% H₃PO₄ Chromosorb WAW column (Supelco, Inc., Bellefonte, Pa.).

Electron microscopy. Cells of fructose- or H₂-CO₂-grown *C. ljungdahlii* were allowed to settle onto carbon-coated Formvar film on copper electron microscope grids. For whole-cell transmission electron microscopy, cells were fixed with glutaraldehyde or left untreated and then negatively stained with phosphotungstic acid or uranyl acetate or left unstained. Electron micrographs were obtained with a Zeiss model EM-10 transmission electron microscope. Energy-dispersive spectroscopy (12) of unstained fructose- or H₂-CO₂-grown cells was performed with a JOEL model 2000 transmission electron microscope equipped with a Delta 4 Quantum thin-window X-ray detector (Kevex, San Carlos, Calif.).

16S rRNA sequence analysis. Cells of *Clostridium barkeri* ATCC 25849, *Clostridium lituseburense* ATCC 25759, *Clostridium pasteurianum* ATCC 6013, and *Clostridium tyrobutyricum* ATCC 25755 were provided by John L. Johnson. The 16S rRNA sequences of *C. barkeri*, *C. lituseburense*, *C. ljungdahlii*, *C. pasteurianum*, and *C. tyrobutyricum* were determined in the laboratory of Carl R. Woese (17, 20). The 16S rRNA sequences of *Bacillus subtilis* and *Escherichia coli* have been published previously (3, 13). Sequences were analyzed by a distance matrix analysis by using a program for fitting trees to distance matrix data, as adapted by workers in the laboratory of Carl R. Woese (7, 16, 20).

Analytical procedures. Growth was determined by measuring turbidity in aluminum seal tubes (1). DNA from *C. ljungdahlii* was purified, and the G+C content was deter-

* Corresponding author.

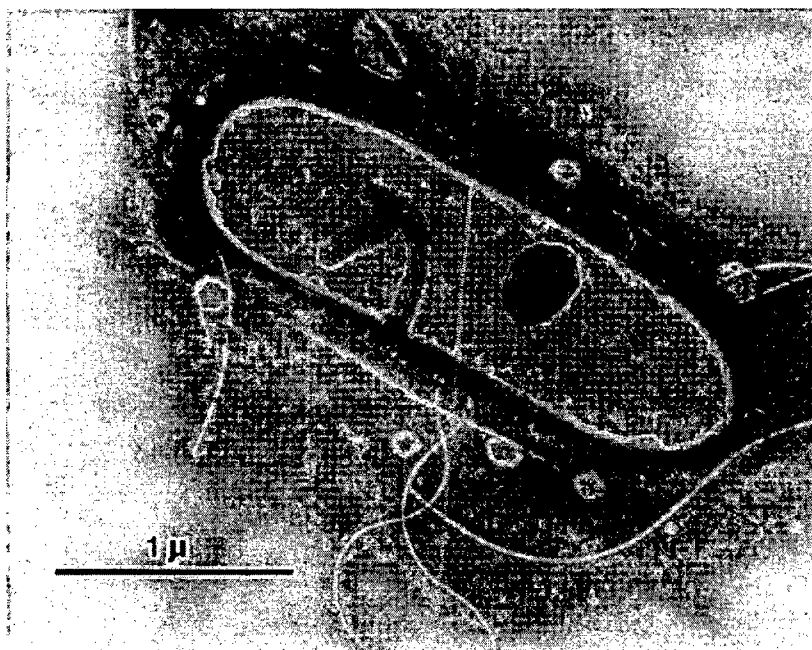


FIG. 1. Transmission electron micrograph of *C. ljungdahlii*. Cells were spread on a carbon-coated Formvar grid and negatively stained with 1% phosphotungstic acid (pH 7). The micrograph was taken with a Zeiss model EM-10 transmission electron microscope.

mined from the melting temperature by using a Beckman model DU-8B spectrophotometer equipped with a melting temperature module (14). Indole production and esculin hydrolysis were determined by using standard assays (21).

Nucleotide sequence accession numbers. The nucleotide sequences of *C. barkeri*, *C. lituseburens*, *C. ljungdahlii*, *C. pasteurianum*, and *C. tyrobutyricum* were deposited in the GenBank library under accession numbers M23927, M59107, M59097, M23930, and M59113, respectively.

RESULTS AND DISCUSSION

Cellular morphology. Electron and phase-contrast microscopy revealed that cells of *C. ljungdahlii* were motile, straight rods (0.6 by 2 to 3 μm) occurring mostly as single cells (Fig. 1). Spores were rarely observed; terminal to subterminal nonswelling structures tentatively identified as forespores were observed infrequently. Cells were peritrichous and had a thick (0.1- to 0.2- μm) coat of material of unknown composition surrounding each cell. Electron microscopy of *C. ljungdahlii* was problematic. The majority of cells in unstained or stained specimens that were not fixed or were fixed with glutaraldehyde were electron opaque. Energy-dispersive spectroscopy (Fig. 2) indicated that these electron-opaque cells contained calcium, as shown by the X-ray peaks at 3.7 keV ($\text{K}\alpha_1$) and 4.0 keV (K edge) (12). The calcium was probably associated with the cell envelope, since lysed cells also were frequently opaque to transmission electron microscopy (data not shown).

Physiology. *C. ljungdahlii* grew autotrophically with H_2 - CO_2 or CO and used a number of organic compounds, including ethanol, pyruvate, and some simple carbohydrates, as carbon and energy sources (Table 1). Growth on formate was poor. Cultures partially metabolized malate, as indicated by a pH change in the medium after incubation with this substrate. Improved control of culture pH near the

optimum value (see below) might result in better growth of *C. ljungdahlii* with formate or malate. Methanol, even when tested at a concentration of 1 g/liter, as well as the methoxylated compounds ferulic acid and trimethoxybenzoate, did not support growth.

Addition of a vitamin solution (22) was required for growth of *C. ljungdahlii*. Addition of yeast extract (1 g/liter) or Casamino Acids (2 g/liter) was also required for reliable, reproducible growth. The exact nutritional requirements of *C. ljungdahlii* were not determined.

The optimum initial culture pH for growth was 6.0; the initial culture pH range at which growth occurred was 4.0 to 7.0. A final pH of 3.9 to 4.1 was obtained when *C. ljungdahlii* was cultured in unbuffered media. The optimum temperature for growth was 37°C; the growth temperature range was 30 to 40°C. Under the optimal pH and temperature conditions, a doubling time of 0.26 h^{-1} was obtained for growth with either fructose or H_2 - CO_2 .

Cultures of *C. ljungdahlii* quantitatively converted 4 mmol of hydrogen and 2 mmol of carbon dioxide into 1 mmol of acetic acid or 1 mmol of fructose into 2.44 mmol of acetic acid; no other end products of metabolism were detected in these experiments. The fermentation balances demonstrate that *C. ljungdahlii* is an acetogen, a bacterium which can synthesize acetate from one-carbon compounds (19). Most acetogens quantitatively convert carbohydrates to acetate: 1 hexose $\rightarrow$ 3 acetic acid. The conversion by *C. ljungdahlii* of 1 fructose molecule to 2.44 acetate molecules is similar to the yields of acetate observed for other acetogens: 2.1 to 2.3 acetate molecules per hexose molecule for *Acetobacterium carbinolicum* (10); 2.2 to 2.8 acetate molecules per fructose molecule for *Acetobacterium woodii* in media supplemented with yeast extract and/or Trypticase (22a); 2.3 to 3.0 (average, 2.5) acetate molecules per glucose molecule for *Acetogenium kivui* (18); an average of 2.55 acetate molecules per