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DEP:	2020 DIRECCION DE NUEVAS CREACIONES	EVE:	379 FASE NACIONAL INCLUIDO CAPITULO II
TRA:	11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS:	6
ACT:	519 PRESENTACION		

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
JUAN PABLO CADENA SARMIENTO

Asunto: Radicación: 14-103917- -1
 Trámite: 11
 Evento: 379
 Actuación: 519
 Oficio: 10400
 Folios: 6

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I		Capítulo II	X
No. Solicitud Internacional	PCT/US2010/002239				
Fecha de Presentación Internacional	13/08/2010				

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Rad. N° 14-103917-00000-0000	14/Mayo/2014
Reivindicaciones:	Rad. N° 14-103917-00000-0000	14/Mayo/2014
Dibujos:	Rad. N° 14-103917-00000-0000	14/Mayo/2014
Prioridades	US 61/233,821	13/Agosto/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10)

reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Por lo tanto se estudiarán las reivindicaciones 1 y 2.

Título de la solicitud: “Aparato y proceso para la fermentación hidrolizada de biomasa” (Pág. 1, radicado N° 14103917-00000-0000).

El problema planteado en esta solicitud consiste en la necesidad de mejorar los procesos de fermentación de un hidrolizado de biomasa para la conversión en biocombustibles.

Para resolver este problema técnico, la solicitud en estudio proporciona un medio de inmovilización que presenta microorganismos que conviertan pentosas.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP) C12P 1/00, 7/10.

4. De la Solicitud de Patente

Título

Se sugiere modificar el título para que quede más acorde al capítulo reivindicatorio de esta solicitud divisional y que incluya la inmovilización que lo caracteriza.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 y 2 no son claras porque los términos “*al menos*” y “*aproximadamente*” son imprecisos, lo que no permite una adecuada comparación con el estado de la técnica. Se sugiere emplear un rango concreto o término definido.

Las reivindicaciones 1 y 2 no son claras porque las expresiones “*cepa de microbio capaz de fermentar las pentosas*” y “*substancia de inmovilización*” son generales, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlas por un término preciso o un rango concreto.

Las reivindicaciones 1 y 2 no son claras porque las expresiones “*es capaz de convertir al menos...hasta un biocombustible*”, “*capaz de proporcionar un micro medio ambiente*” y “*capaz de fermentar las pentosas*” son características funcionales que no le pueden brindar novedad ni nivel inventivo a las reivindicaciones de las cuales dependen. Se sugiere remplazarlas por las características esenciales estructurales que contribuyen a la obtención de ese efecto.

Descripción (Art 28 y Art 29 D 486)

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito en una institución reconocida para los microorganismos recombinantes como lo son las cepas 8b de *Zymomonas mobilis*, *Pichia*, *Candida*, *Kluyveromyces* y *Pachysolen tannophilus*, es decir para aquellos microorganismos que presenten diferentes características fenotípicas con referencia a las del tipo silvestre y sobre las cuales se menciona que presentan modificación genética. Las instituciones reconocidas que son aceptadas para dicho depósito se encuentran en la lista publicada por la OMPI.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad invocada, es decir, el 13 de agosto de 2009.

Consultadas las fuentes de información con que se cuenta, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud.

Item	Documento	Título	Fecha de Publicación
D1	Art 1 ¹	"The performance of serial bioreactors in rapid continuous production of ethanol from dilute-acid hydrolyzates using immobilized cells"	2008
D2	US 4477569	"Pentose fermentation with selected yeast"	16/Octubre/1984

El documento D1 divulga un proceso para transformar hidrolizados de madera en etanol (Pág. 2227, Col. 2, Párr. 3 y Pág. 2228, Col. 1, Párr. 3 a Col. 2, Párr. 1).

El documento D2² divulga un proceso para transformar xilosa en etanol por medio de la levadura *P. tannophilus* (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).

6. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art 18 D486)

La reivindicación 1 consiste en "*Un medio para fermentar el hidrolizado de biomasa, caracterizado porque comprende:...*" (Ver Pág. 3 del Rad. N° 14-103917-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Medio para fermentar el hidrolizado de biomasa, caracterizado porque comprende:	Cultivo continuo de un hidrolizado de madera empleando <i>Saccharomyces cerevisiae</i> encapsulada (Pág. 2227, Col. 2, Párr. 3 y Pág. 2228, Col. 1, Párr. 3 a Col. 2, Párr. 1).	Conversión fermentativa en etanol de la D-xilosa en donde <i>P. tannophilus</i> se inmoviliza en geles de alginato de Ca (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).
Perlas de alginato de calcio	En un cultivo continuo se realiza la fermentación del hidrolizado empleando <i>S. cerevisiae</i> inmovilizada en alginato de calcio (Pág. 2228, Col. 1, Párr. 2 a Col. 2, Párr. 1).	<i>P. tannophilus</i> se inmoviliza en geles de alginato de Ca (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).
que varían de desde 0.1 mm hasta 5 mm de diámetro; y	La esferas tiene un tamaño de 3.5 mm (Pág. 2229, Col. 1, Párr. 1).	No se menciona
Una cepa de microbio capaz de fermentar las pentosas inmovilizadas en las perlas de alginato de calcio.	No se menciona	Se tiene <i>P. tannophilus</i> que se inmoviliza en geles de alginato de Ca (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).

Los documentos D1 y D2 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 1, porque divulgan procesos de fermentación para la obtención de un bioproducto.

La diferencia entre la invención, definida en la reivindicación 1 y el medio del documento D1 consiste en que D1 no menciona la cepa de microbio que fermenta pentosas.

¹Purwadi R. y Taherzadeh MJ. Bioresource Technology.2006. Vol 99.Págs. 2226 a 2233.

²<http://www.google.com/patents/US4477569>

La diferencia entre la invención, definida en la reivindicación 1 y el medio del documento D2 consiste en que en D2 no se menciona el diámetro de las perlas de alginato.

El efecto que logra el incluir la cepa de microbio que fermenta pentosas es que permite la obtención de un biocombustible a partir de medios que tiene pentosas.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así “¿cómo modificar el medio de D1 para que se lleve a cabo la obtención de un biocombustible a partir de pentosas de forma más eficiente?”.

Sin embargo, la conversión fermentativa en etanol de la D-xilosa en donde *P. tannophilus* se inmoviliza en geles de alginato de Ca, ya está divulgado en el documento D2 que se refiere a la producción de un bioproducto a partir de carbohidratos (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).

En consecuencia, una persona normalmente versada en la materia incluiría la cepa de *P. tannophilus* de D2 en el medio de la anterioridad D1, esperando con certeza lograr el producto como el de la reivindicación 1 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

La reivindicación 2 consiste en “*Un medio para fermentar el hidrolizado de biomasa, caracterizado porque comprende:...*” (Ver Pág. 3 del Rad. N° 14-103917-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Medio para fermentar el hidrolizado de biomasa, caracterizado porque comprende:	Cultivo continuo de un hidrolizado de madera empleando <i>Saccharomyces cerevisiae</i> encapsulada (Pág. 2227, Col. 2, Párr. 3 y Pág. 2228, Col. 1, Párr. 3 a Col. 2, Párr. 1).	Conversión fermentativa en etanol de la D-xilosa en donde <i>P. tannophilus</i> se inmoviliza en geles de alginato de Ca (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).
Una sustancia de inmovilización capaz de proporcionar un medio ambiente; y	En un cultivo continuo se realiza la fermentación del hidrolizado empleando <i>S. cerevisiae</i> inmovilizada en alginato de calcio (Pág. 2228, Col. 1, Párr. 2 a Col. 2, Párr. 1).	<i>P. tannophilus</i> se inmoviliza en geles de alginato de Ca (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).
Una cepa de microbio capaz de fermentar las pentosas	No se menciona	Se tiene <i>P. tannophilus</i> que se inmoviliza en geles de alginato de Ca (Col. 1, líneas 1 a 2 y 9 a 11, Col. 3, líneas 60 a 61).
En donde la cepa de microbio comprende aproximadamente 5% en volumen de la sustancia de inmovilización.	Se tienen 100 ml de la suspensión de células que se mezclan con 250 ml de la solución de alginato (pág. 2228, Col. 1, Párr. 2).	No se menciona

Los documentos D1 y D2 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 2, porque divulgan procesos de fermentación para la obtención de un bioproducto.

La diferencia entre la invención, definida en la reivindicación 2 y el medio del documento D1 consiste en que D1 no menciona la cepa de microbio que fermenta pentosas.

La diferencia entre la invención, definida en la reivindicación 2 y el medio del documento D2 consiste en que en D2 no se menciona el volumen de las células en alginato.

No se aprecia efecto al tener un 5% de microbio en relación a la sustancia de inmovilización porque la solución al presente problema ya se encuentra divulgado en el documento D2.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así “¿cómo modificar el medio de D2 para que se lleve a cabo la obtención de un biocombustible a partir de pentosas de forma más eficiente?”.

Sin embargo, células inmovilizadas con una relación a un volumen de alginato, ya está divulgado en el documento D1 que se refiere a la producción de un bioproducto a partir de carbohidratos (Pág. 2227, Col. 2, Párr. 3 y Pág. 2228, Col. 1, Párr. 3 a Col. 2, Párr. 1).

En consecuencia, una persona normalmente versada en la materia incluiría por estrategia de optimización un volumen definido de células en alginato como por ejemplo el mencionado en D1 en el medio de la anterioridad D2, esperando con certeza lograr el producto como el de la reivindicación 2 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

7. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Art 1	1 y 2	Nivel inventivo
D2	US 4477569	1 y 2	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 sesenta (60) días contados a partir del día siguiente 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.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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 No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-08-19

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The performance of serial bioreactors in rapid continuous production of ethanol from dilute-acid hydrolyzates using immobilized cells

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Abstract

The performance of single, and series of, continuous stirred-tank (CSTBR) and fluidized-bed bioreactor (FBBR) in anaerobic continuous cultivation of glucose in defined media and dilute-acid hydrolyzates at dilution rates 0.22, 0.43, 0.65 and 0.86 h⁻¹ using immobilized *Saccharomyces cerevisiae* CBS 8066, was investigated. While the single CSTBR and FBBR could not take up more than 77% and 92% of glucose in a defined medium at dilution rate 0.86 h⁻¹, addition of the second bioreactor decreased the residual glucose to less than 1.1% of the incoming sugar. A similar trend was obtained in cultivation of dilute-acid hydrolyzates. A CSTBR could take up 75% and 54% of the initial fermentable sugars at dilution rates 0.43 and 0.86 h⁻¹, while the addition of the FBBR improved the assimilation of the sugars to 100% and 86%, respectively. The ethanol yields from the hydrolyzate were between 0.41 and 0.48 g/g in all the experiments. The specific and volumetric ethanol productivities were 1.13 g/g h and 5.98 g/L h for the single bioreactor and 0.98 g/g h and 5.49 g/L h for the serial bioreactor at the highest dilution rate, respectively. Glycerol was the only important by-product in terms of concentration, and yielded 0.05–0.07 g/g from the hydrolyzate. From the initial 3.98 g/L acetic acid present in the hydrolyzate, 0.1–0.8 g/L was assimilated by the cells. The yeast cells were accumulated close to the surface of the beads. While the cells had a dry-weight concentration of 129 g/L close to the surface of the beads, the concentration in the core was only 13 g/L.

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Keywords: Serial bioreactor; *S. cerevisiae*; Dilute-acid hydrolyzate; Ethanol; Continuous fermentation

1. Introduction

Production of ethanol from lignocellulosic materials has been a subject of research and development in many countries in the last decades. The major motivation for this subject has been the desire for access to an alternative renewable liquid fuel from local lignocellulosic resources. The process involves principally chemical and/or enzymatic hydrolysis of lignocellulose to the corresponding sugars followed by fermentation to ethanol. Dilute-acid hydrolysis is one of the suitable methods for either chemical hydrolysis or the pretreatment step prior to enzymatic hydrolysis (Sivers and Zacchi, 1995; Sun and Cheng, 2002).

Fermentation in batch is the most common and easiest mode of operation. However, this method cannot succeed well in cultivation of wood hydrolyzates. It is most likely the result of toxic compounds such as furans, phenolic compounds and/or carboxylic acids, which are released during the hydrolysis of lignocelluloses (Larsson et al., 1999; Taherzadeh et al., 1997). The fermentation was improved by application of fed-batch technique (Taherzadeh et al., 1999). In this technique, the concentrations of the inhibitors are controlled in the media by regulation of the feeding. On the other hand, the continuous mode of cultivation has generally been of interest to minimize the total time of fermentation (Turton et al., 2003) as well as decrease the investment cost (Loukatos et al., 2003). Furthermore, since the bioreactor is fed continuously, it would be possible to avoid inhibition to the yeast caused

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Nomenclature

a_{in}, a_{out}	the amount of A entering and leaving the reactor (g)	T	time (s, h)
k	first order reaction constant (h^{-1})	W	washout function (–)
K_s	monod constant (g/L)	Y_A	fraction of escaping molecules A (–)
N	number of tanks in series	μ	specific growth rate (g/L h)
R	bead radius (m)	μ_{max}	maximum specific growth rate (h^{-1})
S	substrate concentration (g/L)	τ	residence time (s, h)

by high concentration of the inhibitors. However, if the inflow of the inhibitors exceeds the capacity of the yeasts in conversion of the inhibitors, the continuous cultivation fails and the biomass is washed out. Taherzadeh et al. (2001) showed that continuous fermentation of a fermentable dilute-acid hydrolyzate can be successful at dilution rate 0.1 h^{-1} , but fails at dilution rates 0.2 h^{-1} or higher. However, they could not cultivate hydrolyzates with higher toxicity even at very low dilution rates.

Detoxification of the hydrolyzates can be carried out chemically or enzymatically, where the toxic materials are converted to non-toxic or less toxic components (Mussatto and Roberto, 2004; Palmqvist and Hahn-Hagerdal, 2000a). Detoxification by lime is one the most practical detoxification methods (Martinez et al., 2001; Millati et al., 2002; Purwadi et al., 2004) due to its simplicity and adequate results. On the other hand, since the cells can detoxify the hydrolyzates *in situ* (Horvath et al., 2001; Liu et al., 2004; Taherzadeh et al., 2000a,b), high cell content in the bioreactor is an advantage for the fermentation process. The cells can be retained inside the bioreactor by different methods such as cell recycling or immobilization. Cell immobilization has an advantage in handling contamination since cells are attached to the matrix while the contaminants are not, therefore the separation of contaminants can be carried out easier. A combination of prior detoxification and cell immobilization can lead to successful fermentation of the hydrolyzates, even at high dilution rates (Taherzadeh et al., 2001). It helps in rapid fermentation of the hydrolyzates.

Continuous cultivation with immobilized cells at high dilution rates in traditional CSTR bioreactors has some drawbacks. The first problem is damaging of the cell bead due to the mixing of the culture with mechanical stirrers. In order to overcome this problem, other types of bioreactors should be applied, such as packed-bed or fluidized-bed bioreactors, where the beads stay packed or fluidized in the culture. A packed-bed bioreactor may have some disadvantages in terms of clogging the bioreactor, through the effect of incoming particles or high local concentration of the inhibitors at the entrance of the bioreactor. In fluidized-bed bioreactor, the beads might be fluidized by entering gas or recirculation of the culture medium. The second problem in continuous cultivation with immobilized cells refers to the inverse correlation between sugar assimilation

and dilution rate (Godia et al., 1987). At high dilution rates, the assimilation declines and a significant part of sugars is washed out. Using serial bioreactor could be a solution to this problem (Tyagi and Ghose, 1980; Tzeng and Fan, 1991). Although it was proved that the series of free cell bioreactors does not have better performance compared to a single bioreactor because of wash-out limitation, the combination of immobilization technique and serial bioreactor improves the sugar assimilation (Gooijer et al., 1996).

In the present study, continuous cultivation of dilute-acid hydrolyzate by immobilized *Saccharomyces cerevisiae* was investigated using serial bioreactors including a continuous stirred-tank bioreactor (CSTBR) and a fluidized-bed bioreactor (FBBR). The capabilities of this system in fermentation of glucose and the hydrolyzates were compared with a single CSTR bioreactor. In addition, the performance of the beads in cell retention was investigated.

2. Methods

2.1. Wood hydrolysis and detoxification

Spruce forest residuals were used in preparing dilute-acid hydrolyzates in this work. The residuals were hydrolyzed in a two-stage hydrolysis using a masonite gun batch reactor. In the first stage, wood, water and acid were mixed to obtain 5 g/L sulfuric acid and 30% (w/w dry-weight) solid concentrations. The mixture was then hydrolyzed by direct steaming at 12 bar pressure and 10 min retention time. The residual solids were then filtered and hydrolyzed at the second-stage at 21 bar for 7 min. Since the hydrolyzates were toxic for direct fermentation, they were then detoxified by addition of calcium hydroxide to raise the pH to 11.0, keep it at this pH at room temperature for 1 h, and then neutralize it by sulfuric acid to achieve pH 5.0 (Purwadi et al., 2004). The calcium salts were precipitated by natural sedimentation and the clear solution was used for cultivation. The detoxification condition was preferred to ensure the fermentability of hydrolyzates even though up to 30% of sugar was lost due to detoxification. The composition of this detoxified hydrolyzate was in g/L: galactose 2.72, glucose 7.24, mannose 18.43, xylose 8.11, acetic acid 3.98, furfural 0.31 and hydroxymethyl furfural

0.56. The hydrolyzates were directly used for cultivation after separation of solid calcium salts by precipitation.

2.2. Yeast strain and medium

Saccharomyces cerevisiae CBS 8066, obtained from Centraalbureau voor Schimmelcultures (Delft, the Netherlands), was used in all experiments. The strain was maintained on agar plates made from 10 g/L of yeast extract, 20 g/L of soy peptone, and 20 g/L of D-glucose as an additional carbon source. Inoculum cultures were grown in 250 mL cotton-plugged conical flasks on a shaker bath at 30 °C for 24 h. The liquid volume was 100 mL and the growth medium was a defined synthetic medium as previously reported (Taherzadeh et al., 1997).

2.3. Immobilization procedure

A 100-mL inoculum culture including 5 g/L biomass was mixed with a 250 mL sterilized solution of Na-alginate to give 350 mL of 3% alginate. The inoculum-alginate mixture was then dropped aseptically through a syringe needle (1.2 × 50 mm) into 30 g/L CaCl₂ solution by a peristaltic pump. The formed beads of the alginate were kept for 2 h to complete the hardening process prior to cultivation (Taherzadeh et al., 2001).

2.4. Cultivation conditions

Anaerobic continuous cultivation was carried out by immobilized *S. cerevisiae* in two bioreactors, including a continuous stirred-tank bioreactor (CSTBR) and a fluidized-bed bioreactor (FBBR), either individual or in series. The CSTBR was a 2 L Biostat-A bioreactor (B.Braun Biotech, Germany), while the FBBR was a locally made 1.3 L bioreactor with 60 mm-ID and 46 cm length connected to the control units of a BIOLAB bioreactor (B.Braun Biotech). The cultivations in both of the bioreactors were carried out in 1 L liquid volume at 30 °C, pH 5.0, and continuously purged with 500 mL/min nitrogen gas. The CSTBR was mixed gently at 150 rpm except for bead performance determination where 200 and 300 rpm mixing speed were used. The lower mixing rate was preferred to minimize the destruction of the beads. The gas purging provided enough energy to fluidize the beads in FBBR. The gas flow was controlled by a mass flow controller (Bronkhorst, the Netherlands). Peristaltic pumps (Watson-Marlow Alitea AB, Sweden) were applied to feed and remove the effluent of the bioreactors. The ethanol concentration was calibrated due to ethanol evaporation in the gas effluent. The calibration was based on the rate of ethanol evaporation from ethanol solution at various concentrations placed in the bioreactor with the fermentation condition.

The experiments were carried out in a completely defined medium (Taherzadeh et al., 1997) with either glucose or dilute-acid hydrolyzates as the carbon and energy

sources. All the continuous cultivations were started by aerobic batch cultivations with the media containing 50 g/L glucose in order to prepare a high concentration of the biomass inside the beads. They were then turned to anaerobic continuous cultivation by addition of the medium containing either 20 g/L glucose or hydrolyzates at various dilution rates (D) of 0.22, 0.43, 0.65 and 0.86 h⁻¹. The experiments were duplicated and the relative standard deviations among the results did not exceed 3.9%.

2.5. Analytical methods

Samples for high-performance liquid chromatography analysis were withdrawn from the bioreactor, centrifuged and stored at -4 °C. Glucose, xylose, galactose and mannose were analyzed on an ion-exchanged Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) at 85 °C. Ultrapure water was used as the eluent at a flow rate of 0.6 mL/min. An evaporative light scattering detector (Waters 2465, Massachusetts, USA) was used to determine the sugar concentrations. Ethanol, glycerol, acetic acid, succinic acid, pyruvic acid, HMF and furfural were analyzed by an Aminex HPX-87H column (Bio-Rad) at 60 °C with 5 mM H₂SO₄ eluent at 0.6 mL/min. An ultraviolet absorbance (UV) detector (Waters 2486) in series with a refractive index (RI) detector (Waters 2410) were used, and all component concentrations were determined from the RI chromatogram except HMF and furfural, which were determined from the UV chromatogram at 210 nm wavelength.

2.6. The profile of cell density inside the bead

The profile of the cell concentration in the beads was obtained according to the following procedure. At the end of the continuous cultivations 10 beads were separated from the bioreactors, and weighed and diluted in 2 mL 0.1 M Na-citrate. After 2–4 min, the beads were separated from the solution, re-weighed and re-diluted in the fresh Na-citrate solution. The cell concentration in the solution was determined by using a spectrophotometer at 620 nm, through correlation of turbidity calibrated by cell dry-weight concentration.

2.7. Determination of number of cells and cell vitality

The number of the cells inside the bead was determined by diluting 10–12 beads in 4 mL 0.1 M Na-citrate. The diameter and weight of the bead were also measured prior to the dilution. The supernatant was then mixed with 2% Rhodamine solution, in the ratio of 20:1, respectively. The total cell number per unit volume was determined using a Burkner counting chamber. The vital cells were distinguished from the dead cells by their color difference, since the vital cells had a rigid wall and therefore the pigment could not be absorbed, while the dead cell absorbed this pigment. The vitality of the cells was calculated on the basis of these colorless cells.

3. Results

3.1. Characterization of the beads

The cultivations in the current work were carried out by immobilized *Saccharomyces cerevisiae* in Ca–alginate beads. The beads had a fairly uniform size with diameter of 3.5 ± 0.2 mm. There were about 14,000 beads/L of fermentation medium, with a total bead volume of approximately 315 ± 10 mL. These beads were used in a continuous stirred-tank bioreactor with moderate mixing of either 200 or 300 rpm to determine the performance of the beads. A slurry of $\text{Ca}(\text{OH})_2$ was used to control the pH and to provide enough calcium ions for maintaining the Ca–alginate matrix.

The weight and size of the beads were monitored during continuous cultivation of the hydrolyzates in continuous stirred-tank bioreactors (CSTBR), as was the number of the cells inside the beads. The weight of the beads decreased gradually during the cultivation due to attrition and breakage. In operation with the higher stirring rate (300 rpm), a total of 30% initial bead weight was lost in 7 days, where the bead sizes decreased from 3.5 to 3.1 mm in diameter. On the other hand, when the speed of the stirrer was reduced to 200 rpm, the weight loss of the beads was recorded as 24% in 11 days and the average bead sizes were reduced from 3.5 mm to 3.2 mm in diameter. In contrast, the cell density inside the beads was increased faster when the mixing rate was at 300 rpm, where it increased by 2.5 times in 7 days compared to 1.7 times in 11 days at the mixing rate of 200 rpm.

3.2. Continuous cultivation on glucose

The immobilized *S. cerevisiae* in Ca–alginate beads were used for cultivation of 20 g/L glucose in a completely defined medium, using a CSTBR and a fluidized-bed bioreactor (FBBR) individually and in series. A summary of the

results on glucose conversion, volumetric ethanol productivity, concentration of glucose and ethanol as well as ethanol yield is shown in Table 1. The experiments were carried out at several dilution rates from 0.22 up to 0.86 h^{-1} , where each experiment lasted for at least five retention times in order to reach steady state.

The cells were able to take up all the glucose in CSTBR, while the bioreactor was fed at dilution rate 0.22 h^{-1} . However, increasing the dilution rate up to 0.86 h^{-1} resulted in increasing the residual glucose in the outflow of the bioreactor up to 23% of the initial sugar or 4.81 g/L (Table 1). The continuous cultivation in FBBR at the lower dilution rate of 0.22 h^{-1} was not as good as in CSTBR, where 2% of the sugars were not assimilated by the yeast. On the other hand, FBBR had a much better performance than CSTBR at the high dilution rate of 0.86 h^{-1} , in which 92% of the sugars were assimilated (Table 1).

The addition of FBBR to CSTBR in the serial arrangement has improved the performance of the cultivation significantly. While the CSTBR could take up only 90% and 77% of the glucose at 0.64 and 0.86 h^{-1} , the additional FBBR could take up the residual sugar and leave zero and one percent of the sugars at these dilution rates, respectively (Table 1).

The ethanol yield in either the individual or serial bioreactors was obtained between 0.43 and 0.49 g/g, while the volumetric ethanol productivity varied with the dilution rates. In the single CSTBR bioreactor, volumetric ethanol productivity increased from 2.15 g/L h at dilution rate 0.22 h^{-1} up to 6.55 g/L h at dilution rate 0.86 h^{-1} (Table 1). However, increase in the volumetric ethanol productivity was not in linear correlation with the dilution rate. The highest volumetric ethanol productivity was obtained in the continuous cultivation in FBBR at dilution rate 0.86 h^{-1} as 7.39 g/L h.

Glycerol was the only important by-product in terms of concentration. The yield of glycerol was almost constant

Table 1

Continuous cultivation of glucose in synthetic medium using immobilized *S. cerevisiae* in single continuous stirred-tank bioreactor (CSTBR), single fluidized-bed bioreactor (FBBR), and combination of these bioreactors in series

$D \text{ (h}^{-1}\text{)}$	Glucose*		Ethanol		Yields of by-products (g/g)			
	Assimilation (%)	Concentration (g/L)	Yield (g/g)	Productivity (g/L h)	Glycerol	Acetic acid	Succinic acid	Pyruvic acid
<i>Single CSTBR</i>								
0.22	100.0	0	0.49	2.15	0.06	0.002	0.002	0.001
0.43	97.7	0.48	0.49	4.19	0.06	0.002	0.002	0.001
0.65	90.2	2.01	0.48	5.71	0.05	0.003	0.002	0.001
0.86	77.2	4.81	0.47	6.55	0.05	0.003	0.002	0.002
<i>Serial CSTBR – FBBR</i>								
0.65	100.0	0	0.47	3.14	0.05	0.002	0.002	0.001
0.86	98.9	0.23	0.47	4.21	0.05	0.003	0.002	0.002
<i>Single FBBR</i>								
0.22	97.7	0.45	0.47	2.00	0.06	0.002	0.003	0.002
0.86	92.0	1.69	0.44	7.39	0.06	0.003	0.003	0.002
<i>Serial FBBR – CSTBR</i>								
0.22	100.0	0	0.45	0.97	0.06	0.002	0.002	0.002
0.86	100.0	0	0.43	3.95	0.06	0.002	0.002	0.002

* The initial glucose concentration was 20 g/L.

between 0.05 and 0.06 g/g regardless of the dilution rate and the type of the bioreactor. The yields of acetic, pyruvic and succinic acids were also monitored, and were all less than 0.003 g/g (Table 1).

3.3. Cultivation of hydrolyzate using single and serial bioreactor

The dilute-acid hydrolyzate was continuously cultivated by the immobilized cells of *S. cerevisiae* located in CSTBR as well as in the serial bioreactor of CSTBR followed by FBBR. The most important results are summarized in Table 2 and Fig. 1. The experiments were carried out at different dilution rates of 0.22, 0.43, 0.65 and 0.86 h⁻¹. Among the sugars contained in the hydrolyzate, glucose, mannose and galactose were consumed, while the concentration of xylose remained unchanged in the bioreactors.

The individual CSTBR could successfully ferment the hydrolyzate at all the dilution rates. However, the concentrations of the residual sugars were higher than in the corresponding experiments with the glucose in synthetic media. The cells were able to assimilate 97.7% of the fermentable sugars at dilution rate 0.22 h⁻¹, while they could not succeed in taking up more than 54.4% of the sugars at dilution rate 0.86 h⁻¹ (Table 2). However, looking at the individual sugars, while glucose, mannose and galactose were present in the medium, the cells assimilated 71.6%, 50.9% and 35.3% of glucose, mannose and galactose respectively (Fig. 1). Addition of a FBBR to the CSTBR in a serial bioreactor system has increased the assimilation of the fermentable sugars and decreased the residual sugars. At the low dilution rate of 0.22 h⁻¹, there was no fermentable sugar left in the outlet of the second bioreactor, and only 13.4% of the sugars were left at the high dilution rate of 0.86 h⁻¹ (Table 2 and Fig. 1).

The volumetric ethanol productivities in both the single and serial bioreactor were increased by increasing the dilution rate (Table 2). However, the maximum productivities obtained at the highest dilution rate of 0.86 h⁻¹ for the sin-

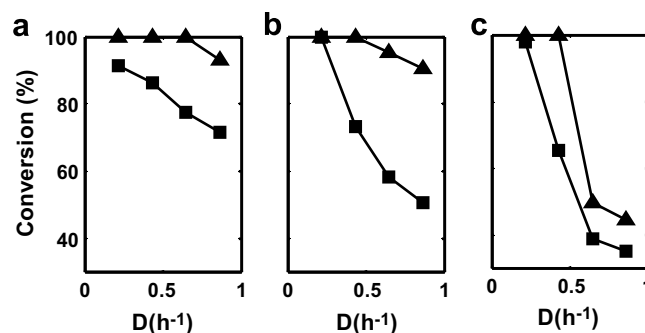


Fig. 1. Conversion of (a) glucose, (b) mannose and (c) galactose in cultivation of dilute-acid hydrolyzates using immobilized *S. cerevisiae* in individual CSTBR (■) and series of CSTBR and FBBR (▲).

gle and serial bioreactor were of the same order of magnitude, i.e. 5.98 corresponding to 5.49 g/L h, respectively. Accounting for the biomass concentrations of 5.31 and 5.62 g/L for the single and serial bioreactor, the corresponding “specific ethanol productivities” are calculated as 1.13 g/g h for the single and 0.98 g/g h for the serial bioreactor at the highest dilution rate of 0.86 h⁻¹. The ethanol yield at the low dilution rate of 0.22 h⁻¹ was obtained 0.44 and 0.46 for the single and serial bioreactor, while the yield was higher for the serial bioreactor at the higher dilution rates (Table 2).

The yields of glycerol obtained were between 0.049 and 0.068 g/g in all the experiments with dilute-acid hydrolyzates (Table 2), which are of the same order as the corresponding yields from glucose in the synthetic media (Table 1). No formation but consumption of acetic acid present in the hydrolyzates was detected. However, of the 3.98 g/L acetic acid entering the bioreactors, only 0.1 to 0.8 g/L could be assimilated by the yeast. Furfural was totally converted by the yeasts in all the experiments, except with the single bioreactor at the highest dilution rate (Table 2) but the cells were not so successful in converting all the HMF contained in the hydrolyzates. While the single bioreactor system could convert 41.4% of the HMF at

Table 2

Continuous cultivation of dilute-acid hydrolyzate using immobilized *S. cerevisiae* in single continuous stirred-tank bioreactor (CSTBR) and series of CSTBR and fluidized-bed bioreactor (FBBR)

D (h ⁻¹)	Total fermentable sugars		Ethanol productivity (g/L h)	Yields (g/g)		Furan conversion (%)	
	Assimilation (%)	Residual (g/L)		Ethanol	Glycerol	Furfural	HMF
<i>Single CSTBR</i>							
0.22	97.7	0.65	2.70	0.44	0.050	100.0	85.8
0.43	75.0	7.57	4.63	0.45	0.051	100.0	74.5
0.65	61.1	11.96	5.20	0.43	0.065	100.0	47.6
0.86	54.4	14.02	5.98	0.41	0.068	96.2	41.4
<i>Serial CSTBR – FBBR</i>							
0.22	100.0	0.00	1.42	0.46	0.049	100.0	93.4
0.43	100.0	0.00	2.84	0.48	0.050	100.0	85.9
0.65	92.0	2.45	4.43	0.48	0.059	100.0	78.8
0.86	86.6	4.11	5.49	0.48	0.064	100.0	75.3

The initial total concentration of the fermentable sugars (glucose, mannose and galactose) was 28.4 g/L.

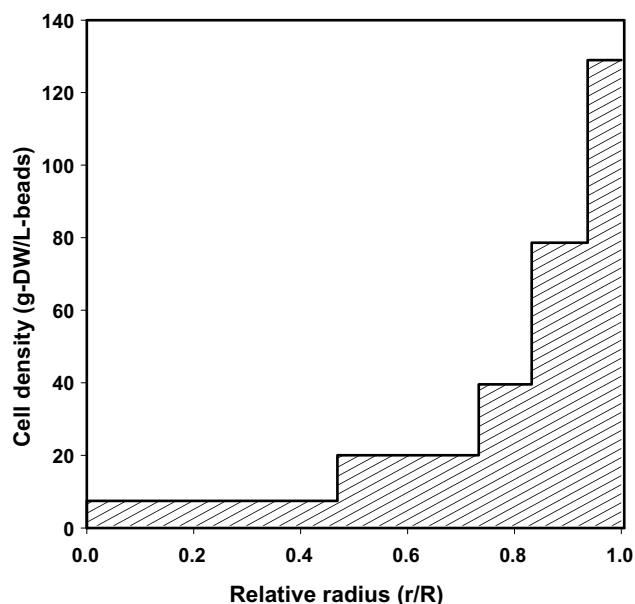


Fig. 2. Profile of cell density at different positions inside the bead. The beads were used for hydrolyzate cultivation for 2 weeks at dilution rate of 0.43 h^{-1} . “R” is radius of the bead, while “r” is the distance of the measured point from the center of the bead.

the highest dilution rate of 0.86 h^{-1} , the addition of the second bioreactor could increase the HMF conversion to 75.3% (Table 2).

The cell concentration at the surface of the beads was much higher than in their core. At dilution rate 0.86 h^{-1} , the average dry-weight cell concentration was 38.18 g/L of the beads. However, the cell concentration was not constant throughout the beads. The profile of the cell concentration was measured for the beads that had been in use for two weeks with dilution rate 0.43 h^{-1} . While the cell concentration in the 5% of the radius toward the surface was measured as 129 g/L , the average cell concentration in the interior 40% of the radius was measured as 13 g/L (Fig. 2). The vitality of the cells inside the beads was also examined by using staining with Rhodamine pigment. The results show that the cell vitality was more than 90% in all experiments.

4. Discussion

Continuous cultivation with retained cells in serial bioreactor is a promising method for rapid cultivation of wood hydrolyzates. The results of the current work show the advantages of immobilization as well as application of serial bioreactors in continuous cultivation of dilute-acid hydrolyzates at high dilution rate. No similar work was found in the literature. However, serial bioreactors have been used to reduce the residual sugars at low dilution rate. Tzeng and Fan (1991) succeeded with assimilation of 82% of glucose in cultivation of 200 g/L glucose, in an approximately 8-stage fluidized-bed bioreactor with total volume of 410 mL at flow rate of 95.7 mL/h , which is equivalent

to dilution rate 0.23 h^{-1} . In another work, Tyagi and Ghose (1980) fermented bagasse hydrolyzate in a 4-stage stirred-tank reactor with suspended cells. They reported 20%, 71% and 100% for the first, second and third reactor at dilution rate of 0.2 h^{-1} . This could be compared with our result that the fermentable sugars in the hydrolyzates were completely converted at dilution rate 0.43 h^{-1} in the serial bioreactor. Furthermore, with high concentration of cells entrapped in the beads (5.98 g/L), the specific ethanol productivity of single and serial immobilization bioreactor system respectively were 1.13 and 0.98 g/g h at dilution rate of 0.86 h^{-1} . As a comparison, Rudolf et al. (2004) performed fermentation of undetoxified hydrolyzate using fed-batch bioreactor and they reached specific ethanol productivity of 0.9 g/g h .

Since fermentation can be considered an autocatalytic reaction, a composition of CSTR followed by PFR is the best arrangement of the reactors, where CSTR is an increasing reaction rate zone while PFR is a decreasing reaction rate zone (Trambouze and Euzen, 2004). Due to the operational problems in working with cultivation of the hydrolyzates, it might be difficult – especially in aerobic processes – to design an ideal tubular plug-flow reactor. However, it is possible to approximate this plug-flow reactor by a chain of stirred-tank reactors. The volume optimization of the bioreactor which is employed for continuous cultivation using cell suspension can be carried out graphically, through the plot of reciprocal rate of substrate consumption versus substrate concentration which was demonstrated by Gooijer et al. (1996). In this system, the reaction rate reaches a maximum point where the most favorable size of tank reactor can be achieved. At the lower substrate concentration, the substrate consumption rate decreases due to the loss of the cells (washout). In contrast, a fermentation system with immobilized cells keeps the cells inside the reactor; therefore active material is always available. If the cell washout can be avoided, the flow rate is not limited by the washout, but probably by the elapsed time of the molecules inside the reactor and by the reaction rate.

At low dilution rate in glucose cultivation, there was enough time for substrate molecules to be converted, whereas at higher dilution rates the substrate partially washed out due to lack of reaction time. The washout behavior is a non-ideality of real reactor and has been explained in many reactor handbooks (Nauman, 2001; Trambouze and Euzen, 2004). The washout function, $W(t)$, is defined as the fraction of molecules leaving the system that have experienced a residence time greater than t . The mathematical descriptions of washout function for single and multiple stirred-tanks are:

$$\text{Single: } W(t) = e^{-t/\tau} \quad (1)$$

$$\text{Multiple: } W(t) = \frac{N^N t^{N-1}}{(N-1)! \tau^{N-1}} e^{-Nt/\tau} \quad (2)$$

where τ is residence time. These equations show that the serial tank reactor could provide longer residence time and

therefore facilitate the reaction to increase the conversion. Moreover, for the first-order reaction, the fraction of escaping molecules A , Y_A , can be described as:

$$Y_A = \frac{a_{\text{out}}}{a_{\text{in}}} = 1 - k \int_0^\infty e^{-kt} W(t) dt \quad (3)$$

It is clearly seen that even though the washout function provides adequate effect on the conversion, the kinetic of the reaction is still considerable. Obviously, these factors work in concert to increase the conversion.

In general, anaerobic fermentation by *S. cerevisiae* can be regarded as exhibiting constant overall reaction stoichiometry where segregation of bioreactor volume may lead to a higher product concentration, a higher degree of conversion and a higher volumetric productivity (Gooijer et al., 1996). The Monod kinetic is often used as a relationship of specific growth rate to substrate concentration:

$$\mu = \frac{\mu_{\text{max}} S}{K_s + S} \quad (4)$$

where μ_{max} is the maximum specific growth rate when $S \gg K_s$. When $\mu = \mu_{\text{max}}$, the rate becomes zero-order reaction and, theoretically, there is no difference in conversion between a serial reactor and a single reactor with equal total reaction volume. In addition, if $S \ll K_s$ then $\mu = (\mu_{\text{max}}/K_s) \cdot S$ and the rate becomes first-order reaction. In this case, the conversion is slightly higher in a serial reactor than a single reactor. This behavior is clearly seen in the results of glucose cultivation (Table 1). The comparison should be in equal residence time; for example, the glucose assimilation in serial reactor at dilution rate of 0.86 h^{-1} should be compared with the glucose assimilation in single reactor at dilution rate of 0.43 h^{-1} , since the serial reactor has double volume capacity relative to the single reactor. The results show that segregation had only gained about 1.2% of glucose assimilation in synthetic medium. However, this behavior was not observed in cultivation of the hydrolyzate. The assimilation of fermentable sugars (hexoses) in hydrolyzate cultivation was much lower than the glucose assimilation in glucose cultivation at the same dilution rate (Table 2). Obviously, since the hydrolyzate contains various components and some of them are inhibitory to the yeast, the hexoses uptake rate slows down. Furfural and HMF are the most inhibitory compounds generated from acid hydrolysis of lignocelluloses (Horvath et al., 2001; Palmqvist and Hahn-Hagerdal, 2000b; Taherzadeh et al., 2000a,b), and it was shown that the yeast takes up the furans as an alternative to the sugars. Furthermore, Modig et al. (2002) proposed the kinetic model of these furan inhibition reactions, which was categorized as a mixed non-competitive/competitive model of inhibition. The first bioreactor suffers more inhibition and converts less sugar while the second bioreactor bears less inhibitor thus has a capacity to convert more sugar. Thus the mechanism reaction seems to be heterogeneous reactions where serial bioreactor is superior to single bioreactor.

The hexoses uptake rate was also influenced by the individual uptake rate of fermentable sugars: glucose, mannose and galactose. Fig. 1 shows the conversion of each sugar in hydrolyzates at different dilution rates. The glucose was the fastest sugar taken up by the yeast, followed by mannose and galactose. All these phenomena result in lower rates of cultivation of the hydrolyzate compared to the synthetic medium. Therefore, the application of a serial reactor for hydrolyzate cultivation offered a great benefit. The reactor segregation gained about 11.6% of hexose assimilation and this was substantial compared to glucose cultivation.

The fermentable sugar concentration was rather low due to detoxification process where about 30% of sugar was lost. The detoxification condition was chosen to ensure the fermentability of hydrolyzates. Even though the ethanol yields were in the adequate ranges, but the ethanol concentrations produced were quite low due to this sugar concentration. The low sugar content would affect the downstream process such as separation and purification. The detoxification condition could be milder and the concentration of fermentable sugar could be higher even though care should be taken to maintain the fermentability of hydrolyzates.

The cell density profile (Fig. 2) shows that most of the cells were sited in the outer layer of the bead. This result was comparable with other similar works (Laca et al., 2000, 1998; Wang et al., 1995) although the determination of the density profile was quite different. One parameter affecting the cell density profile was the distribution of substrate through the beads since the cells need the substrate to grow. The substrate profile can be found through determination of the Thiele modulus (Fogler, 1992; Laca et al., 1998), which is defined as the ratio of a surface reaction rate to a rate of diffusion through the bead matrix. Due to difficulties of measuring the reaction rate at the surface, which also depended on the concentration of biomass and local kinetic parameters, the overall reaction rate could be used with some allowance for error. With the determined effective diffusivity and bead diameter of 3.5 mm, the Thiele modulus for hydrolyzate cultivation was calculated (details not shown) and the values obtained were 4.18 at dilution rate 0.22 h^{-1} decreasing to 1.39 at dilution rate 0.86 h^{-1} . If the value of the Thiele modulus is large, then the diffusion limits the overall rate of reaction and the substrate does not easily pass the matrix; hence the biomass is concentrated close to the surface of the beads. Whenever the value of the Thiele modulus is small enough, the substrate passes through the bead matrix and supplies enough substrate to the inner cells to grow. As a result, a flat biomass profile should be obtained, which was not the case in the current work.

It can be concluded that the serial bioreactor was superior to the single bioreactor in cultivation of dilute-acid hydrolyzates with immobilized cells at high dilution rates. It gives high productivity for ethanol and leaves low residual concentration of the sugars in the outflow of the bioreactors.

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