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TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 6
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

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

Asunto: Radicación: 14-103917- -3
 Trámite: 11
 Evento: 379
 Actuación: 519
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 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° 14-103917-00002-0000	19/Noviembre/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 a 4.

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.

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

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 las expresiones “*cepa microbiana fermentativa*” y “*medio 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 concisas porque el término “*cepa microbiana fermentativa*” involucra todos los microorganismos existentes en la naturaleza, los cuales no se encuentran en su totalidad soportados en la descripción de la solicitud y que presentan algún efecto técnico al estar inmovilizados y tratados con un medio de cultivo.

Por lo tanto el capítulo reivindicatorio no es claro ni conciso porque hace referencia a cualquier microorganismo, siendo el objeto de la solicitud la inmovilización de microorganismos como *Zymomonas mobilis*, *Pichia*, *Candida*, *Kluyveromyces* y *Pachysolen tannophilus*, inmovilización destinada al procesamiento de biomasa hidrolizada.

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.

Ítem	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
D3	Art 2 ²	"Immobilized viable microbial cells: from the process to the proteome. . . or the cart before the horse"	2004
D4	US 2405861	"Production of alginate solutions"	13/Agosto/1946

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 D3 divulga técnicas de inmovilización que favorecen la fermentación (Pág. 640, Tabla 4).

El documento D4³ divulga mejoramiento en la obtención de soluciones de alginato (Col.1, líneas 1 a 9).

6. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 2 consiste en *"Un medio para fermentar el hidrolizado de biomasa, caracterizado porque comprende..."* (Ver Pág. 11 del Rad. N° 14-103917-00002-0000).

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

Características esenciales	D3
Medio para fermentar el hidrolizado de biomasa, caracterizado porque comprende:	Técnicas de inmovilización que favorecen la fermentación (Pág. 640, Tabla 4).
Un medio de inmovilización; y	Se presenta alginato de calcio como medio de inmovilización (Pág. 639, Tabla 3).
Una cepa microbiana fermentativa inmovilizada en el medio de inmovilización,	Se presentan microorganismos inmovilizados en alginato de calcio (Pág. 639, Tabla 3).
en donde la eficiencia metabólica de la cepa microbiana inmovilizada ha sido restaurada mediante el tratamiento de la cepa microbiana inmovilizada en un medio de cultivo mientras la cepa microbiana se encuentra inmovilizada en el medio de inmovilización.	Se presenta un rejuvenecimiento de las células inmovilizadas con un medio rico en nutrientes para aumentar la eficiencia de la fermentación (Pág. 637, Párr. 6 a Pág. 638, Párr. 2).

De acuerdo con la tabla anterior, las características esenciales del medio de la reivindicación 2 ya habían sido divulgadas previamente en el documento D3, por lo tanto, la materia reivindicativa no es nueva.

Así mismo, el documento D3 divulga las características de las reivindicaciones dependientes, así:

Reivindicaciones 3 y 4: diferentes sustratos de inmovilización como alginato de calcio y agarosa, entre otros (Pág. 640, Tabla 4).

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

² Junter GA and Jouenne T. Biotechnology Advances. 2004. Vol. 22. Págs. 633 a 658.

³ <http://www.google.com/patents/US2405861>

Nivel inventivo (Art 18 D486)

La reivindicación 1 consiste en “*Un medio para fermentar el hidrolizado de biomasa, caracterizado porque comprende:...*” (Ver Pág. 11 del Rad. N° 14-103917-00002-0000).

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

Características esenciales	D1	D4
Medio para fermentar 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).	Mejoramiento en la obtención de soluciones de alginato (Col.1, líneas 1 a 9).
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).	No se menciona
que varían 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 microbiana fermentativa inmovilizadas en las 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).	No se menciona
En donde el alginato de calcio empleado para hacer las perlas comprende alginato reciclado.	No se menciona Se presenta degradación de las perlas de alginato de calcio con ayuda de citrato de sodio (pág. 2228, Col. 2, Párr. 3 y Párr. 4).	Se presentan agentes secuestrantes de calcio del alginato de calcio, como el citrato de sodio, para la obtención de alginato de sodio y su uso en formulaciones farmacéuticas (Col. 1, línea 36 a Col. 3, línea 7).

Los documentos D1 y D4 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 1, porque divulgan procesos de que involucran alginato de calcio.

La diferencia entre la invención, definida en la reivindicación 1 y el medio del documento D1 consiste en que D1 no menciona el alginato reciclado.

La diferencia entre la invención, definida en la reivindicación 1 y el medio del documento D4 consiste en que en D4 no se mencionan las perlas ni la cepa microbiana inmovilizada.

El efecto que logra el incluir el alginato reciclado para la obtención de las perlas de alginato de calcio es el de disminuir la materia prima requerida para la fabricación de dichas perlas.

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 disminuya el

consumo de materia prima de forma más eficiente?”.

Sin embargo, la desestabilización del alginato de calcio con agentes secuestrantes como el citrato de sodio para su empleo como alginato de sodio, ya está divulgado en el documento D4 que se refiere a mejoramiento en la obtención de soluciones de alginato (Col. 1, línea 36 a Col. 3, línea 7).

En consecuencia, una persona normalmente versada en la materia incluiría cualquier alginato de sodio como el obtenido a partir de alginato de calcio de D4 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.

Asimismo se tienen etapas de purificación del alginato de sodio obtenido de diversas fuentes cuando se requiere para diversas aplicaciones, entre ellas precipitaciones, lavado con agua y/o solventes y diálisis^{4,5}

7. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Art 1	1	Nivel inventivo
D3	Art 2	2 a 4	Novedad/Nivel inventivo
D4	US 2405861	1	Nivel inventivo

8. Respuesta a los argumentos de la solicitante

En referencia a los argumentos de la solicitante según el cual la solicitud presenta efectos sorprendentes con respecto a los documentos D1 y D2 mencionados en el oficio N° 10400, notificado por fijación en lista el 26 de agosto de 2014, y por lo tanto es patentable, esta Oficina encuentra nuevos documentos D3 y D4 que afectan la patentabilidad de la presente solicitud. Adicionalmente, se reitera la falta concisión en el capítulo reivindicatorio y que se requiere el certificado de depósito de material biológico de los microorganismos que presentan sustento en la descripción, que hace posible la ejecución de dicha solicitud con fines del tratamiento de biomasa hidrolizada.

⁴ Gómez et al, Influence of the extraction–purification conditions on final properties of alginates obtained from brown algae (*Macrocystis pyrifera*). International Journal of Biological Macromolecules. Febrero 2009. Vol. 44. Pág. 366, Fig. 1.

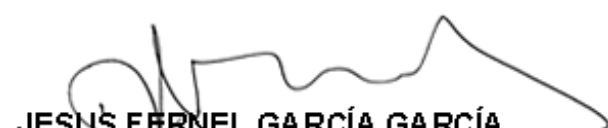
⁵ Calafiore et al. Standard Technical Procedures for Microencapsulation of Human Islets for Graft into Nonimmunosuppressed Patients With Type 1 Diabetes Mellitus. Transplantation Proceedings. 2006. Vol. 38. Abstract.

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.



JESUS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-12-16

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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-

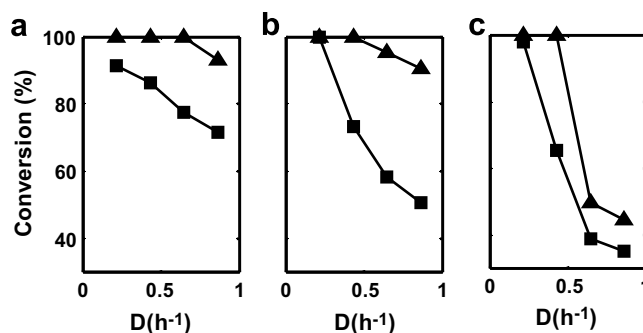


Fig. 1. Conversion of (a) glucose, (b) mannose and (c) galactose in cultivation of dilute-acid hydrolyzates using immobilized *S. cerevisiae* in individual CSTBR (■) and series of CSTBR and FBBR (▲).

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.

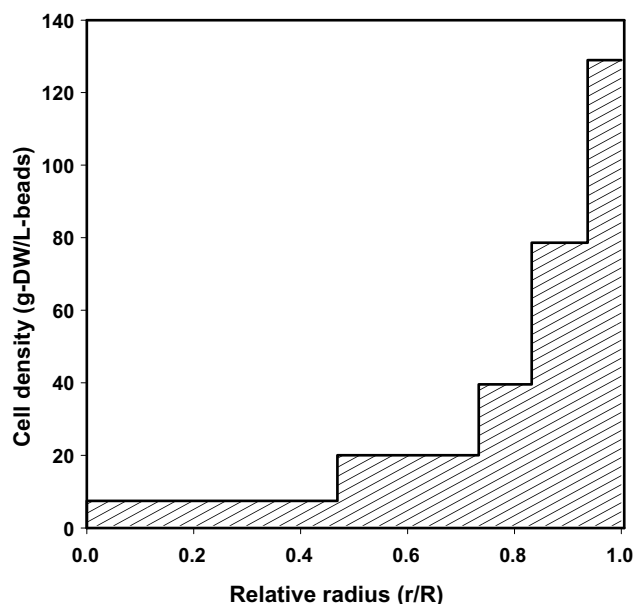


Fig. 2. Profile of cell density at different positions inside the bead. The beads were used for hydrolyzate cultivation for 2 weeks at dilution rate of 0.43 h^{-1} . “R” is radius of the bead, while “r” is the distance of the measured point from the center of the bead.

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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References

- Fogler, H.S., 1992. Elements of Chemical Reaction Engineering, second ed. Prentice-Hall, Inc., New Jersey.
- Godia, F., Casas, C., Sola, C., 1987. A survey of continuous ethanol fermentation systems using immobilized cells. *Process Biochemistry* 4, 43–48.
- Gooijer, C.D.d., Bakker, W.A.M., Beftink, H.H., Tramper, J., 1996. Bioreactors in series: An overview of design procedures and practical applications. *Enzyme and Microbial Technology* 18, 202–219.
- Horvath, I.S., Taherzadeh, M.J., Niklasson, C., Liden, G., 2001. Effects of furfural on anaerobic continuous cultivation of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* 75, 540–549.
- Laca, A., Garcia, L.A., Diaz, M., 2000. Analysis and description of the evolution of alginate immobilized cells systems. *Journal of Biotechnology* 80, 203–215.
- Laca, A., Quiros, C., Garcia, L.A., Diaz, M., 1998. Modelling and description of internal profiles in immobilized cells systems. *Biochemical Engineering Journal* 1, 225.
- Larsson, S., Palmqvist, E., Hahn-Hagerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.-O., 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme and Microbial Technology* 24, 151–159.
- Liu, Z.L., Slininger, P.J., Dien, B.S., Berhow, M.A., Kurtzman, C.P., Gorsich, S.W., 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. *Journal of Industrial Microbiology and Biotechnology* 31, 345–352.
- Loukatos, P., Kanellaki, M., Komaitis, M., Athanasiadis, I., Koutinas, A.A., 2003. A new technological approach proposed for distillate production using immobilized cells. *Journal of Bioscience and Bioengineering* 95, 35–39.
- Martinez, A., Rodriguez, M.E., Wells, M.L., York, S.W., Preston, J.F., Ingram, L.O., 2001. Detoxification of dilute acid hydrolysates of lignocellulose with lime. *Biotechnology Progress* 17, 287–293.
- Millati, R., Niklasson, C., Taherzadeh, M.J., 2002. Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolysates for fermentation by *Saccharomyces cerevisiae*. *Process Biochemistry* 38, 515–522.
- Modig, T., Liden, G., Taherzadeh, M.J., 2002. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochemical Journal* 363, 769–776.
- Mussatto, S.I., Roberto, I.C., 2004. Alternatives for detoxification of dilute-acid lignocellulosic hydrolysates for use in fermentative processes: a review. *Bioresource Technology* 93, 1–10.
- Nauman, E.B., 2001. Chemical Reactor, Design, Optimization and Scaleup. McGraw Hill, New York.
- Palmqvist, E., Hahn-Hagerdal, B., 2000a. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresource Technology* 74, 17–24.
- Palmqvist, E., Hahn-Hagerdal, B., 2000b. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology* 74, 25–33.
- Purwadi, R., Niklasson, C., Taherzadeh, M.J., 2004. Kinetic study of detoxification of dilute-acid hydrolysates by $\text{Ca}(\text{OH})_2$. *Journal of Biotechnology* 114, 187.
- Rudolf, A., Galbe, M., Liden, G., 2004. Controlled fed-batch fermentations of dilute-acid hydrolysate in pilot development unit scale. *Applied Biochemistry and Biotechnology*, 113–116.
- Sivers, M.v., Zacchi, G., 1995. A techno-economical comparison of three processes for the production of ethanol from pine. *Bioresource Technology* 51, 43–52.
- Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology* 83, 1–11.
- Taherzadeh, M.J., Eklund, R., Gustafsson, L., Niklasson, C., Liden, G., 1997. Characterization and fermentation of dilute-acid hydrolysates from wood. *Industrial & Engineering Chemistry Research* 36, 4659–4665.
- Taherzadeh, M.J., Gustafsson, L., Niklasson, C., Liden, G., 2000a. Inhibition effects of furfural on aerobic batch cultivation of *Saccharomyces cerevisiae* growing on ethanol and/or acetic acid. *Journal of Bioscience and Bioengineering* 90, 374–380.
- Taherzadeh, M.J., Gustafsson, L., Niklasson, C., Liden, G., 2000b. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* 53, 701–708.
- Taherzadeh, M.J., Millati, R., Niklasson, C., 2001. Continuous cultivation of dilute-acid hydrolysates to ethanol by immobilized *Saccharomyces cerevisiae*. *Applied Biochemistry and Biotechnology* 95, 45–57.
- Taherzadeh, M.J., Niklasson, C., Liden, G., 1999. Conversion of dilute-acid hydrolysates of spruce and birch to ethanol by fed-batch fermentation. *Bioresource Technology* 69, 56–66.
- Trambouze, P., Euzen, J.-P., 2004. Chemical Reactors: From Design to Operation. Institut Francais du Petrole Publications, Paris.
- Turton, R., Bailie, R.C., Whiting, W.B., Shaeiwitz, J.A., 2003. Analysis, Synthesis, and Design of Chemical Processes, second ed. Pearson Education, Inc., New Jersey.
- Tyagi, R.D., Ghose, T.K., 1980. Batch and multistage continuous ethanol fermentation of cellulose hydrolysate and optimum design of fermentor by graphical analysis. *Biotechnology and Bioengineering* 22, 1907–1928.
- Tzeng, J.W., Fan, L.-S., 1991. Ethanol fermentation using immobilized cells in a multistage fluidized bed bioreactor. *Biotechnology and Bioengineering* 38, 1253–1258.
- Wang, H., Seki, M., Furusaki, S., 1995. Mathematical model for analysis of mass transfer for immobilized cells in lactic acid fermentation. *Biotechnology Progress* 11, 558–564.



Research review paper

Immobilized viable microbial cells: from the process to the proteome. . . or the cart before the horse

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Abstract

Biotechnological processes based on immobilized viable cells have developed rapidly over the last 30 years. For a long time, basic studies of the physiological behaviour of immobilized cells (IC) have remained in the shadow of the applications. Natural IC structures, i.e. biofilms, are being increasingly investigated at the cellular level owing to their definite importance for human health and in various areas of industrial and environmental relevance. This review illustrates this paradoxical development of research on ICs, starting from the initial rationale for IC emergence and main application fields of the technology—with particular emphasis on those that exploit the extraordinary resistance of ICs to antimicrobial compounds—to recent advances in the proteomic approach of IC physiology.

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Keywords: Biofilm; Bioprocess; Cell physiology; Gel entrapment; Protein expression; Proteomics

Contents

1. Introduction: development and main application fields of IC cultures	634
2. The original motivation of viable IC technology.	636

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3.	Current data on IC physiology.	638
3.1.	Growth rate.	638
3.2.	Biocatalytic efficiency and enzyme expression.	639
3.3.	Stress resistance.	640
4.	The proteomic approach and the biofilm phenotype	644
5.	Conclusion.	652
	References	653

1. Introduction: development and main application fields of IC cultures

Immobilized cell (IC) technologies have widely developed since the early 1980s (Fig. 1A), and thousands of documents concerning ICs are currently available via scientific search websites such as Scirus (Elsevier). Therefore, a number of immobilization procedures have been detailed over the last 20 years, in particular in books, some of which are listed here as examples (Mattiasson, 1983a; Rosevear et al., 1987, Tampion and Tampion, 1987; Veliky and McLean, 1993; Bickerstaff, 1997; Wijffels, 2001). Very briefly, IC systems can be separated into wholly artificial and naturally occurring ones. In the first category, microbial (or eucaryotic) cells are artificially entrapped in or attached to various matrices/supports where they keep or not a viable state, depending on the degree of harmfulness of the immobilization procedure. Polysaccharide gel matrices, more particularly Ca-alginate hydrogels (Gerbsch and Buchholz, 1995), are by far the most frequently used materials for harmless cell entrapment. Cell attachment to an organic or inorganic substratum may be obtained by creating chemical (covalent) bonds between cells and the support using cross-linking agents such as glutaraldehyde or carbodiimide. This immobilization procedure is generally incompatible with cell viability. The spontaneous adsorption of microbial cells to different types of carrier gives natural IC systems in which cells are attached to their support by weak (non-covalent), generally non-specific interactions such as electrostatic interactions. In suitable environmental conditions, this initial adsorption step may be followed by colonization of the support, leading to the formation of a biofilm in which microorganisms are entrapped within a matrix of extracellular polymers they themselves secreted. Owing to the presence of this polymer paste, biofilms are more firmly attached to their substratum than merely adsorbed cells. Hence, they offer more practical potentialities than the latter as IC systems. However, surface colonization to form biofilms is a universal bacterial strategy for survival, and undesirable biofilms may occur on inert or living supports in natural or biological environments as well as in industrial installations. The definite importance of biofilms in various areas of industrial relevance and for human health has been only relatively recently recognized: the last 10 years have known a burst in the number of published investigations on these natural IC systems (Fig. 1B).

As illustrated by Fig. 1 and detailed in Table 1, a large part of published data on artificial or natural IC systems concerns their operation in bioreactors where they perform

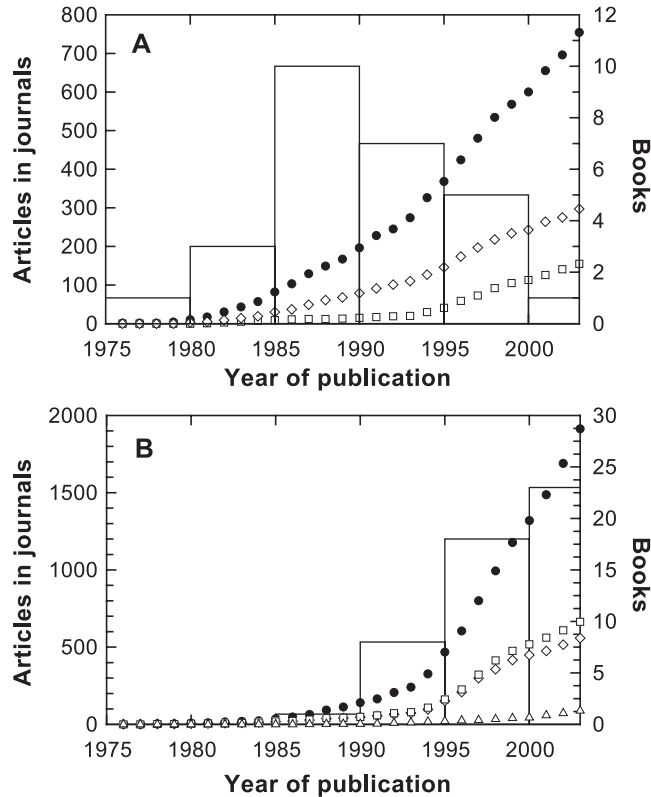


Fig. 1. Time evolution of the number of scientific publications on ICs over the last 30 years. Cumulative numbers of published papers were obtained by consulting the journals database at the Elsevier ScienceDirect website. Histograms were constructed from books recorded in electronic libraries (amazon.com and barnesandnoble.com websites). Key words used for search: (A) immobilized cell: (●) overall; (◇) IC+reactor/bioreactor; (□) IC+degradation/biodegradation, water and wastewater treatment. (B) Biofilm: (●) overall; (◇) biofilm+reactor/bioreactor; (□) biofilm+degradation/biodegradation, water and wastewater treatment; (△) biofilm+antibiotic/resistance.

biosyntheses or bioconversions leading to a variety of compounds, ranging from primary metabolites to high-value biomolecules. IC cultures have also been widely applied to the treatment of domestic or industrial wastewaters containing different types of pollutants such as nitrate/nitrite ions, heavy metals or organic compounds recalcitrant to biodegradation. Together with brewing and winemaking processes, biosensors for environmental monitoring, food quality analysis and fermentation process control complete the main application fields of ICs. Faced with these dominant and prolific developments, research on the physiological behaviour of microbial cells in the immobilized state remains paradoxically limited.

Complementing a previous paper that surveyed recent data on IC physiology (Junter et al., 2002a), the present review underlines this paradoxical development of research on ICs, where practical applications have preceded more fundamental investigations of microbial

Table 1
Main application fields of IC cultures

<i>Biosyntheses, bioconversions</i>	
Enzymes	α -Amylases, cellulase and other cellulolytic enzymes, chitinolytic enzymes, cyclodextrin glucosyltransferase, L-glutaminase, inulase, lipases, penicillin V acylase, peroxidases, polymethylgalacturonase, alkaline and acid proteases, pullulanases, ribonuclease, xylanase
Antibiotics	Ampicillin, candicidin, cephalosporin C, clavulanic acid, cyclosporin A, daunorubicin, divercin, kasugamycin, nikkomycin, nisin Z, oxytetracyclin, patulin, penicillin G, rifamycin B
Steroids ^a	Androstenedione, hydrocortisone, prednisolone, progesterone
Amino acids	Alanine, arginine, aspartic acid, cysteine, glutamic acid, phenylalanine, serine, tryptophan
Organic acids	Acetic, citric, fumaric, gluconic, lactic, malic, propionic acids
Alcohols	Butanol, ethanol, sorbitol, xylitol
Polysaccharides	Alginate, dextran, levan, pullulan, sulfated exopolysaccharides
Varia	Pigments, vitamins, flavors and aroma
<i>Environment</i>	
Water treatment	Carbon removal (COD), nitrogen removal (nitrification/denitrification, assimilation), heavy metal removal (Au, Cd, Cu, Ni, Pb, Sr, Th, U, . . .), pollutant biodegradation (phenol and phenolic compounds, polycyclic aromatics, heterocycles, cyanide compounds, surfactants, hydrocarbons, oily products)
Biofertilisation	Soil inoculation with plant growth-promoting organisms (<i>Azospirillum brasilense</i> , <i>Bradyrhizobium japonicum</i> , <i>Glomus deserticola</i> , <i>Pseudomonas fluorescens</i> , <i>Yarrowia lipolytica</i>)
Bioremediation	Degradation of pollutants in contaminated soils (e.g. chlorinated phenols), aquifers and marine habitats (e.g. petroleum hydrocarbons) by microbial inocula
Alternative fuels	Dihydrogen and methane productions, ethanol production, biofuel cells
<i>Food processing</i>	
Alcoholic beverages	Brewing, vinification, fermentation of cider and kefir; controlled in situ generation of bioflavors
Milk products	Continuous inoculation of milk (lactic starters), lactose hydrolysis in milk whey
<i>Biosensors</i>	
Electrochemical ^b	Acetic acid, acrylonitrile, amino acids, BOD, cyanide, cholesterol, chlorinated aliphatic compounds, ethanol, naphthalene, nitrate, phenolic compounds, phosphate, pyruvate, sugars, sulfuric acid (corrosion monitoring), uric acid, herbicides, pesticides, vitamins, toxicity assays
Optical	Herbicides, metals, genotoxicant, polyaromatics, toxicity testing

^a Obtained by conversion of steroid parent compounds.

^b Amperometric, potentiometric, conductometric.

behaviour in the immobilized state. Recent advances of the proteomic approach concerning both artificial (gel entrapped) and natural (biofilm) IC systems are also presented.

2. The original motivation of viable IC technology

Whole cell immobilization procedures originated from those applied to extracted enzymes some years earlier and the first attempts involved cells impaired by physical and/

or chemical treatment, i.e. nonviable cells, to perform single-step enzyme reactions (Gestrelus, 1983). The main and obvious benefit derived from the use of whole cells instead of enzymes was to avoid enzyme extraction/purification steps and their consequences on enzyme activity, stability, and cost. Immobilization techniques were rapidly extended to viable cells, however. The main advantages of viable IC cultures over conventional (suspended cell) ones, claimed at the very beginning of this research area, are summarized in Table 2 and briefly analysed below.

- (a) As viable ICs are able to multiply during substrate metabolization while remaining confined (to a certain extent) within the immobilization structure (e.g. the polysaccharide gel matrix of artificially gel-entrapped cells or the glycocalyx of natural biofilm organisms), high cell densities may be expected in IC cultures, leading to high volumetric reaction rates.
- (b) Furthermore, this ability to grow in the immobilized state makes it possible for the regeneration of IC cultures following their operation in hostile incubation conditions such as in a low-nutrient medium or in the presence of toxic compounds.
- (c) The use of biomass attached to or entrapped in particulate carriers ensures efficient biomass retention in the reactor during continuous processes, minimizing cell washout that occurs at high dilution rates and limiting the volumetric conversion capacity of classical, free-cell-based continuous stirred tank reactors (i.e. chemostats). Continuous IC bioreactors can therefore be operated at high load, even when diluted feeds are used: a definite advantage in wastewater treatment (Nicolella et al., 2000), for instance.
- (d) Easier downstream processing, due in particular to facilitated cell/liquid separation, represents another asset of fermentation processes using IC cultures.
- (e) From the outset of IC technology, enhanced operational and storage stabilities have been presented as a key feature for practical development of viable IC systems. These stabilities involve both biological and mechanical characteristics of IC biocatalysts. In order to explain the increase in the biological stability of ICs, Dervakos and Webb (1991) proposed several hypotheses based on IC's ability to grow. Here, biological stabilization meant lengthened operation times and improved resistance to storage periods. Alternate operation of ICs between growth and non-growth conditions, adapted to non-growth-associated productions, periodic rejuvenation of the biocatalyst in nutrient-rich medium, allow to maintain long-term biological activities.

Table 2

Potential advantages of viable IC systems over conventional fermentations: a "historical" point of view (adapted from Vieth and Venkatsubramanian, 1979; Mattiasson, 1983b)

-
- (a) Higher reaction rates due to increased cell densities
 - (b) Possibilities for regenerating the biocatalytic activity of IC structures
 - (c) Ability to conduct continuous operations at high dilution rate without washout
 - (d) Easier control of the fermentation process
 - (e) Long-term stabilization of cell activity
 - (f) Reusability of the biocatalyst
 - (g) Higher specific product yields
-

Cryptic growth from cell debris inside IC structures was also advocated to explain the maintenance of IC activity in nutrient-poor reaction media. The protective effect of the immobilization matrix against physicochemical stresses was also put forward. More recently, [Freeman and Lilly \(1998\)](#) reviewed the effect of processing parameters on the operational stability of aerobic IC cultures, including mechanical behaviour of the IC carrier. These parameters included the immobilization method, the mode of operation (e.g. repeated batch vs. continuous), aeration and mixing, the bioreactor configuration, medium composition, temperature, pH and, if necessary, in situ product and/or excess biomass removal.

- (f) Reusability of IC biocatalysts also depends on the efficiency of rejuvenation periods to maintain the biological activity of ICs and the ability of IC materials to endure both processing stresses and these rejuvenation steps at the mechanical level.
- (g) The last claimed advantage of IC cultures over conventional free-cell ones is an increase in product yield. This is actually the only “historical” feature referring to possible “advantageous metabolic changes” ([Dervakos and Webb, 1991](#)) in ICs. Product yield improvement of IC cultures will be commented on later.

The technological obstacles to a large-scale industrial implementation of IC systems have also been regularly investigated, with particular emphasis on the mass transfer limitations inside immobilization matrices and the coupled transport-reaction phenomena that control the performance of IC cultures ([Karel et al., 1985, 1990](#); [Radovich, 1985](#); [Walsh and Malone, 1995](#); [Pilkington et al., 1998](#); [Riley et al., 1999](#)).

Therefore, it appears that the initial rationale for IC development essentially concerned the engineering level, with very few—if any—queries on the physiological behaviour of microbial cultures in the immobilized state. This historical prevalence of applications over more basic investigations may explain why our present knowledge of IC physiology still remains fragmentary.

3. Current data on IC physiology

3.1. Growth rate

Up to now, the physiological behaviour of ICs has been mainly studied at the macroscopic level by observing changes in metabolic activities in the immobilized state, more particularly by comparing the biocatalytic efficiency of ICs to that of suspended cultures. Microbial growth in the presence of sugars or more specific substrates has also been monitored in (natural or artificial) IC systems. Published results show contradictory effects of (natural or artificial) immobilization on growth rate, i.e. decreased, unchanged or enhanced growth rates of ICs compared to free cultures, as illustrated in [Table 3](#) for a variety of organisms entrapped in calcium alginate gel beads. Mass transfer limitation in IC systems, leading to the formation of nutrient- and/or oxygen-deprived microenvironments, gives the most evident explanation to reduced IC growth rate. On the other hand, the growth-promoting action of immobilization has been attributed to protective effects of the support, e.g. against

Table 3

Reported changes in specific growth rates or doubling times upon immobilization by entrapment in Ca alginate beads

Organism/substrate	Growth parameters ^a	References
<i>Saccharomyces cerevisiae</i> /glucose	$\mu_i=0.25 \text{ h}^{-1}$ $\mu_s=0.41 \text{ h}^{-1}$	Galazzo and Bailey, 1990
<i>Chlamydomonas reinhardtii</i> /CO ₂ +NO ₂ [−]	$t_{di}=9 \text{ h}$ $t_{ds}=8 \text{ h}$	Santos-Rosa et al., 1989
<i>Xanthomonas maltophilia</i> /acrylamide	$t_{di}=8 \text{ h}$ $t_{ds}=4 \text{ h}$	Nawaz et al., 1993
<i>Pseudomonas</i> sp./acrylamide	$t_{di}=6 \text{ h}$ $t_{ds}=2 \text{ h}$	Nawaz et al., 1993
<i>Prototheca zopfii</i>	$\mu_i < \mu_s$	Suzuki et al., 1998
<i>Acinetobacter johnsonii</i> /activated sludge mixed liquor	$\mu_i = \mu_s$	Muyima and Cloete, 1995
<i>Saccharomyces cerevisiae</i> /glucose	$\mu_i=0.30 \text{ h}^{-1}$ $\mu_s=0.31 \text{ h}^{-1}$	Willaert and Baron, 1993
<i>Trichosporon cutaneum</i> /glucose	$t_{di}=3 \text{ h}$ $t_{ds}=4 \text{ h}$	Chen and Huang, 1988
<i>Aspergillus niger</i> /apple pectin	$\mu_i > \mu_s$	Pashova et al., 1999
<i>Acinetobacter calcoaceticus</i> /activated sludge mixed liquor	$\mu_i=2\mu_s$	Muyima and Cloete, 1995

^a t_{di} , t_{ds} , division (generation) times and μ_i , μ_s , specific growth rates of immobilized and suspended (free) cells, respectively.

high-shear environment (Chun and Agathos, 1991) or acidification (Taipa et al., 1993). Chen and Huang (1988) have put forward a better microenvironment at the level of ICs due to the retention of growth-promoting factors in the network of the entrapment matrix.

3.2. Biocatalytic efficiency and enzyme expression

Owing to the industrial importance of yeast cell cultures, a number of studies have focused on the metabolic responses of yeasts to immobilization (Norton and D'Amore, 1994), showing an activation of the energetic metabolism of yeasts upon immobilization, namely increased specific rates of substrate (essentially glucose) uptake and product (essentially ethanol) excretion (Table 4). More generally, enhanced production/conversion efficiencies of ICs as compared to suspended counterparts have been presented at the very beginning as one of the main advantages of IC cultures from a practical point of view (Table 2). Published results are often given on a volumetric scale, however, which is of real interest for biochemical engineers but does not characterize the intrinsic behaviour of ICs. Higher specific production rates and/or yields of ICs than those of suspended organisms have been actually observed, e.g. for the production of secondary metabolites such as enzymes (Klingenberg et al., 1990) and antibiotics (Farid et al., 1995; Azanta Teruel et al., 1997). Conversely, IC cultures have been shown to display unchanged or even lower specific productivities as compared to free-cell cultures, and this in a variety of productions, including enzymes (Abdel-Naby et al., 2000; Longo et al., 1999) and antibiotics (Scott et al., 1988). Mass transfer limitations in IC systems are mainly responsible for this decrease in

Table 4

Physiological responses of *S. cerevisiae* (fed with glucose) to immobilization

Immobilization technique	Metabolic responses	References
Colonization of porous ceramic beads	Increased glycerol production and specific alcohol dehydrogenase activity	Demuyakor and Ohta, 1992
Attachment to cross-linked gelatin	Increased specific rates of glucose consumption and ethanol production. Changes in cellular composition (larger quantities of reserve carbohydrates and structural polysaccharides)	Doran and Bailey, 1986
Entrapment in Ca alginate beads	Increased specific rates of glucose uptake, ethanol and glycerol production; enhanced synthesis of polysaccharide storage materials	Galazzo and Bailey, 1989
Entrapment in agarose beads	Two-fold faster glucose fermentation kinetics	Lohmeier-Vogel et al., 1996
Adsorption to DEAE-cellulose	Higher glucose flux and enhanced excretion of main metabolic products	Van Iersel et al., 2000
Entrapment within oxystarch-hardened gelatin gel disks	Modifications in the pattern of cell wall mannoproteins	Parascandola et al., 1997
Covalent linkage to a hydroxyalkyl methacrylate gel	Enhanced resistance to ethanol accompanied by an alteration in the plasma membrane composition	Jirku, 1999
Entrapment in Ca alginate beads or adsorption on sintered glass rings	Greater ethanol tolerance and fermentation capability; enhanced saturation in total fatty acid composition	Hilge-Rotmann and Rehm, 1991

specific production rates. Biocatalytic efficiency is obviously subject to the biosynthesis of the relevant enzyme systems. Increased specific activities of enzymes in ICs have been highlighted, e.g. β -galactosidase in immobilized *Escherichia coli* (Lyngberg et al., 1999) and superoxide dismutase in *Aspergillus niger* (Angelova et al., 2000). Differences in the specific activities of intracellular enzymes, e.g. alcohol dehydrogenase (Demuyakor and Ohta, 1992; Van Iersel et al., 2000), have also been reported in immobilized yeast cells compared to suspended counterparts. Sonomoto et al. (2000) reported that *Lactococcus lactis* cells adsorbed on chitosan or photo-cross-linked resin gel beads produced nisin Z, a peptide antibiotic, with higher yield and volumetric productivity than free cultures during repeated batch fermentations, whereas opposite results were observed with gel-entrapped organisms. In addition, the production yield of adsorbed cultures was lower than that of suspended ones in continuous experiments. These results illustrate the difficulties in assessing the role of immobilization on intrinsic cellular parameters from chemical engineering data.

3.3. Stress resistance

A major characteristic of ICs is their high resistance to environmental stresses, in particular, the exposure to toxic compounds.

As a key parameter in the performance of alcoholic fermentation by IC cultures, the tolerance of immobilized yeast cells to ethanol is well-documented (Table 4; see also Norton and D'Amore, 1994). Many reports connect this resistance to changes in structural features affecting IC permeability, namely the composition and organization of the cell wall and the plasma membrane (Hilge-Rotmann and Rehm, 1991; Parascandola et al., 1997; Jirku, 1999). Adverse environmental conditions in IC structures, i.e. high osmotic pressure (Hilge-Rotmann and Rehm, 1991) and nutrient limitations and/or mechanical stress (Parascandola et al., 1997) have been advanced to try to explain these modifications in IC permeability.

The biodegradation of toxic compounds, pollutants and xenobiotics also represents a preferential application field of IC systems (Table 1). The high biodegradation efficiency and operational stability of IC cultures, highlighted for instance, during continuous biodegradation assays of phenol and phenolic derivatives (Table 5), is typically ascribed to some protecting effect of the immobilization support (Dervakos and Webb, 1991), rather than to enhanced specific degradation capacity that might involve physiological modifications in ICs. In the case of the widely investigated biodegradation of phenol, several authors have implied reversible adsorption of the pollutant on the immobilization matrix (O'Reilly and Crawford, 1989; Hu et al., 1994; Cassidy et al., 1997; Annadurai et al., 2000) to explain the observed rise in the inhibition threshold of ICs.

ICs are also characterized by a high resistance to antimicrobial agents such as biocides and antibiotics. This resistance has been observed for artificially immobilized microbial cultures, e.g. alginate entrapped bacteria exposed to sanitizers (Trauth et al., 2001) or antibiotics (Coquet et al., 1998), but more frequently for natural IC systems, namely biofilms, which are implied in a variety of industrial, environmental and medical situations. In particular, the reduced susceptibility of biofilm-embedded bacteria to antibiotics (Table 6) is a crucial problem for the treatment of chronic infections such as those associated with implanted medical devices (Stickler and McLean, 1995; Habash and Reid, 1999) or lung infection in cystic fibrosis patients (Singh et al., 2000; Høiby, 2002), and contribute to the occurrence of nosocomial infections (Vuong and Otto, 2002). The reasons for this enhanced resistance of biofilm bacteria to antimicrobials is still a matter of controversy (Costerton et al., 1999; Mah and O'Toole, 2001). In addition to the hindered penetration of inhibitors in the biofilm structure due to diffusional limitations in the so-called glycocalyx, the reduced access of nutrients and/or oxygen to the cell surface and the resulting slow growth rates of organisms, more particularly, those cells that are deeply embedded in the biofilm, may contribute to the lower overall susceptibility of sessile bacteria to many antibiotics, e.g. beta-lactamines and fluoroquinolones (Ashby et al., 1994; Tanaka et al., 1999; Anderl et al., 2003). Nevertheless, these factors linked to restricted diffusion in IC structures are insufficient to explain the loss in antimicrobial efficiency of antibiotics against biofilm organisms (Anderl et al., 2000; König et al., 2001; Stone et al., 2002). Another hypothesis has been advanced recently, assuming the existence of adherence and biofilm phenotypes. Therefore, a variety of bacteria at surfaces and within biofilms have been shown to display altered gene expression as compared to planktonic organisms (Prigent-Combaret et al., 1999; Loo et al., 2000; Whiteley et al., 2001; Schembri et al., 2003). A second way to approach physiological differences between suspended and immobilized microbial cells consists of comparing the amounts of structural components produced in the two culture modes. Proteomics, which focuses on

Table 5
Application of IC cultures to continuous phenol degradation

Microorganisms and Immobilization system	Bioreactor	Operating conditions ^a	Maximum biodegradation rate (mg l ⁻¹ h ⁻¹)	Reusability or service time	References
<i>P. putida</i> , Ca-alginate beads	bubble column (fluidized bed)	100 mg l ⁻¹ mineral salt medium 0.6 h ⁻¹	58.5	n.g. ^b	Mordocco et al., 1999
<i>P. putida</i> , Ca-alginate beads	bubble column (fluidized bed)	1000 mg l ⁻¹ mineral salt medium 0.25–4.0 day ⁻¹	167	3 months	González et al., 2001a
<i>P. putida</i> , Ca-alginate beads	bubble column (fluidized bed)	250–2500 mg l ⁻¹ diluted wastewater 0.25 day ⁻¹	21	60 days	González et al., 2001b
<i>Rhodococcus</i> sp., Ca-alginate beads	packed-bed column	1000 mg l ⁻¹ mineral salt medium 0.086 h ⁻¹	87.5	>6 months	Pai et al., 1995
<i>P. putida</i> + <i>Cryptococcus elinovii</i> , Chitosan-alginate beads	air-lift	1200–3600 mg l ⁻¹ mineral salt medium 0.13–0.31 h ⁻¹	410	>800 h	Zache and Rehm, 1989
<i>Fusarium flocciferum</i> Polyurethane foam cubes	stirred tank	400–1500 mg l ⁻¹ Complex growth medium 0.2 h ⁻¹	200	4 months	Anselmo and Novais, 1992
Mixed culture (from oil-polluted soil), silica gel particles	packed-bed (PB) or fluidized-bed (FB) column	400 mg l ⁻¹ mineral salt medium 0.25–1.65 h ⁻¹	394 (PB), 91 (FB)	n.g.	Brányik et al., 2000

Mixed culture (from oil-polluted soil), polyurethane foam cylinders	packed-bed (PB) or fluidized-bed (FB) column	400 mg l ⁻¹ mineral salt medium 0.25–1.65 h ⁻¹	471 (PB), 161 (FB)	n.g.	Brányik et al., 2000
Acclimated sludge, polyvinyl-alcohol beads	packed-bed column	100 mg l ⁻¹ synthetic wastewater 0.082–1.92 h ⁻¹	179	148 days	Fang and Zhou, 1997
<i>P. putida</i> , Biofilm formation on zeolite-based biocarriers	packed-bed column	1000 mg l ⁻¹ mineral salt medium 1.5–4 day ⁻¹	c15	n.g.	Durham et al., 1994
<i>P. putida</i> , biofilm formation on glass beads	packed-bed column	800 mg l ⁻¹ mineral salt medium 1–4 day ⁻¹	133	≥677 days	Nkhalambayausi- Chirwa and Wang, 2001
<i>Neurospora crassa</i> , biofilm formation on polysulfone capillary membranes	capillary membrane bioreactor module	94–470 mg l ⁻¹ growth medium flow rate, 3 ml h ⁻¹	100 mg m ⁻² h ⁻¹ (1.35 mg g ⁻¹ h ⁻¹)	2 months ^c	Luke and Burton, 2001
<i>Rhodococcus</i> sp., adsorption on granular activated carbon (coconut shells)	packed-bed column	1500 mg l ⁻¹ mineral salt medium 0.086 h ⁻¹	121	≥125 days	Pai et al., 1995

Adapted from Junter et al. (2002b).

^a Phenol concentration in the influent, nature of the treated wastewater, and residence time.

^b n.g., not given.

^c Combining successive exposure and (10-day) recovery periods, preceded by a 2-month operation period in the presence of *p*-cresol.

Table 6
Some examples of increased resistance of attached microorganisms to antibiotics

Organisms	Biofilm substrata	Antibiotics	References
<i>Candida</i> spp.	silicone urinary catheter	amphotericin B, miconazole, ketoconazole, fluconazole, itraconazole	Kalya and Ahearn, 1995
<i>Klebsiella pneumoniae</i>	microporous polycarbonate membrane resting on agar culture medium	ampicillin, ciprofloxacin	Anderl et al., 2000
<i>Mycobacterium smegmatis</i>	polyvinyl chloride dishes	isoniazid	Teng and Dick, 2003
<i>Porphyromonas gingivalis</i>	hydroxyapatite (HA) surfaces	metronidazole	Wright et al., 1997
<i>Porphyromonas gingivalis</i>	membrane filters (modified Robbins device) ^a	amoxicillin, doxycycline and metronidazole	Larsen, 2002
<i>Propionibacterium acnes</i> , <i>Staphylococcus</i> spp.	polymethylmethacrylate (PMMA) bone cement	cefamandole, ciprofloxacin, vancomycin	Ramage et al., 2003
<i>Pseudomonas aeruginosa</i>	latex (urinary) catheter disks	tobramycin	Nickel et al., 1985
<i>P. aeruginosa</i>	silicone disks (modified Robbins device) ^a	fosfomycin, ofloxacin	Kumon et al., 1995
<i>P. aeruginosa</i>	metal studs (modified Robbins device) ^a	ciprofloxacin, tobramycin	Preston et al., 1996
<i>Staphylococcus aureus</i>	fibronectin-coated polymethylmethacrylate cover slips	gentamicin	Chuard et al., 1993
<i>S. aureus</i>	silicone catheter surfaces	tetracycline, benzylpenicillin, vancomycin	Williams et al., 1997
<i>Staphylococcus epidermidis</i>	dacron or teflon vascular grafts	minocycline, cefazolin, vancomycin, rifampin	Bergamini et al., 1996

Susceptibility tests were performed using laboratory (in vitro) models of natural biofilms.

^a In which (metal, plastic, ...) support samples are exposed to the flowing fluid and can be removed aseptically.

gene products as a complementary tool to the gene-level approach, is being increasingly applied to physiological studies of ICs.

4. The proteomic approach and the biofilm phenotype

It emerges from the foregoing that, despite the wealth of published data on ICs and their practical operation in various bioprocesses, despite the well-recognized importance of the immobilized state in microbial way of life and its consequences for human beings, our present knowledge of IC physiology still remains incomplete; in particular, concerning the origins of the extraordinary resistance displayed by ICs to antimicrobial agents. The recent application of proteomic analyses to bacteria in the immobilized state seems a promising approach to try to elucidate the mechanisms underlying the low susceptibility of ICs to antimicrobials, antibiotics, biocides, or toxic pollutants.

Proteomics develops rapidly as a leading route for biological research at the dawn of the post-genomic era. Microbiology sensu lato is one of the major disciplines that are opening up to proteomics-based approaches (Cash, 1998; VanBogelen et al., 1999; O'Connor et al., 2000; Washburn and Yates, 2000; Cash, 2003; VanBogelen, 2003), more particular attention is being paid to medical microbiology as shown by the ever-increasing number of published proteomic analyses concerning pathogens (Wagner et al., 2002; Guina et al., 2003; Hecker et al., 2003; Len et al., 2003; Liao et al., 2003). These investigations have been performed on microorganisms cultured in the suspended mode of growth, wishing to establish protein maps of medically relevant microorganisms, to assess the influence of environmental factors (e.g. stresses) on protein expression, or to elucidate the role of certain gene products in pathogenicity. Nevertheless, this proteomic approach of microbial cell physiology is being extended to ICs, more particularly naturally immobilized (biofilm) organisms—owing to their industrial, environmental and medical implications.

Most proteomic analyses of biofilm cells consists of comparing the crude protein patterns of organisms cultured in the sessile (immobilized) and planktonic (suspended) modes. These studies have revealed some alterations in the bacterial protein profiles ranging from 3% to more than 50% of the detected protein spots (Table 7), which gives evidence of significant physiological differences between the two modes of growth. The complexity of these

Table 7

Number of proteins whose amount was reported to be modified in biofilm cells as compared to planktonic organisms

Microorganism	Biofilm		Number of spots/gel	Number of modified spots ^a		Change (%)	References
	Substratum	Age		+	–		
<i>Bacillus cereus</i>	glass wool fibres	2 h	345	19	4	7	Oosthuizen et al., 2002
		18 h		26	8	10	
<i>Campylobacter jejuni</i>	glass beads	48 h	n.g.	12	7	–	Dykes et al., 2003
<i>Escherichia coli</i>	glass fibre membrane filters	7 days	600	14	3	3	Trémoulet et al., 2002b
<i>E. coli</i>	glass beads	2 h	38 ^b	17	15	84	Otto et al., 2001
<i>Listeria monocytogenes</i>	glass fibre membrane filters	7 days	550	22	9	6	Trémoulet et al., 2002a
<i>P. aeruginosa</i>	glass wool fibres	18 h	844	49	48	11.5	Vilain et al., 2004a
		48 h	838	182	47	27	
<i>P. aeruginosa</i>	clay beads	18 h	816	48	130	22	Vilain et al., 2004a
		48 h	841	62	78	17	
<i>P. aeruginosa</i>	silicone tubing	1 day	≈ 1500	375	60	29	Sauer et al., 2002
		6 days		765	90	57	
<i>Pseudomonas putida</i>	silicone tubing	6 h	1000	15	30	4.5	Sauer and Camper, 2001
<i>Streptococcus mutans</i>	epon-hydroxyapatite rods	3 days	694	57	78	19.5	Svensäter et al., 2001

^a (+) Overproduced; (–) underproduced.

^b Outer membrane proteins.

physiological changes has been highlighted by [Sauer et al. \(2002\)](#), who analysed by two-dimensional gel electrophoresis, four development stages of a *Pseudomonas aeruginosa* biofilm on silicone tubing in a continuous flow reactor: reversible attachment, irreversible attachment, maturation and detachment. The average difference in proteomes between each developmental episode was 35% of detectable proteins. The most profound proteomic alterations were observed in mature biofilm cells (i.e. after incubation for 6 days), with more than 50% of detectable protein spots up-regulated compared to planktonic cells. After longer incubation (12 days), the protein profile of dispersing biofilm cells showed greater similarity to planktonic cells than to 6-day-old biofilm bacteria, with 35% of protein spots down-regulated compared to mature biofilm cells. The authors conclude that attached *P. aeruginosa* cells display multiple phenotypes during biofilm development and that these time-dependent, stage-specific physiologies should be considered for efficient control of biofilm growth.

Proteomic analyses of artificially immobilized bacteria are much scarcer. Polysaccharide gel-entrapped organisms have been shown to represent a simple model structure of natural biofilms ([Jouenne et al., 1994](#)), displaying a low susceptibility to antibiotics similar to biofilms ([Tresse et al., 1995](#); [Coquet et al., 1998](#))—in addition to their well-documented resistance to pollutants as underlined above. The total protein contents of agar-entrapped *E. coli* cells incubated for 2 days in a minimal nutrient medium were compared to those of suspended cells harvested during the exponential or the stationary phase of growth ([Perrot et al., 2000](#)). This 2-DE comparative analysis highlighted noticeable qualitative and quantitative differences in bacterial proteomes according to the incubation conditions, implying about 20% of the total cellular proteins detected on electropherograms (about 790 spots). These results confirm that bacteria cultured as suspended cells undergo physiological changes between the exponential and stationary growth phases, but also shows that gel-entrapped cultures cannot be likened to ordinary stationary-phase cell systems. Using the same immobilization procedure for *P. aeruginosa* cells, [Vilain et al. \(in press\)](#) compared protein expression by suspended and immobilized bacteria after incubation for 18 or 48 h. Once again, noticeable changes (20–25% of detected spots) in protein levels according to the growth mode were revealed by 2-DE. The duration of incubation was shown to exert considerable influence on these modifications. After incubation for 18 h, 114 proteins were overexpressed and 63 underexpressed by ICs. When the duration of incubation was extended to 48 h, the tendency was inverted as the number of underexpressed peptides in ICs (142) largely exceeded that of overexpressed ones (53).

These protein-based approaches to IC physiology, suggesting that many genes are differentially regulated during culture development in the immobilized state, contrast with transcriptome analyses from which only a few genes show altered expression as a consequence of bacterial adhesion ([Whiteley et al., 2001](#); [Schembri et al., 2003](#)). As discussed by [Ghigo \(2003\)](#) in a recent review, however, this modest overlap between results of proteomic and transcriptomic studies is not surprising, since the relationships between mRNA and protein contents are heavily dependent on time, cellular localization and the stability of molecules. Furthermore, the thresholds used to define over- and down-regulations in both transcriptomic and proteomic analyses suffer from the lack of standardization, which may contribute to these discrepancies.

Referring to data reported by Whiteley et al. (2001), however, Hancock (2001) launched a heated debate on the biofilm phenotype, stating that bacteria growing in biofilms are “not that different” from free-living bacteria. A statistical demonstration that bacteria growing in the immobilized state are physiologically different from free-living organisms has been recently published by Vilain et al. (2004a,in press,c). Multivariate methods, more particularly principal component analysis (PCA), were used to interpret the variations in protein spot densities observed on protein maps from *P. aeruginosa*

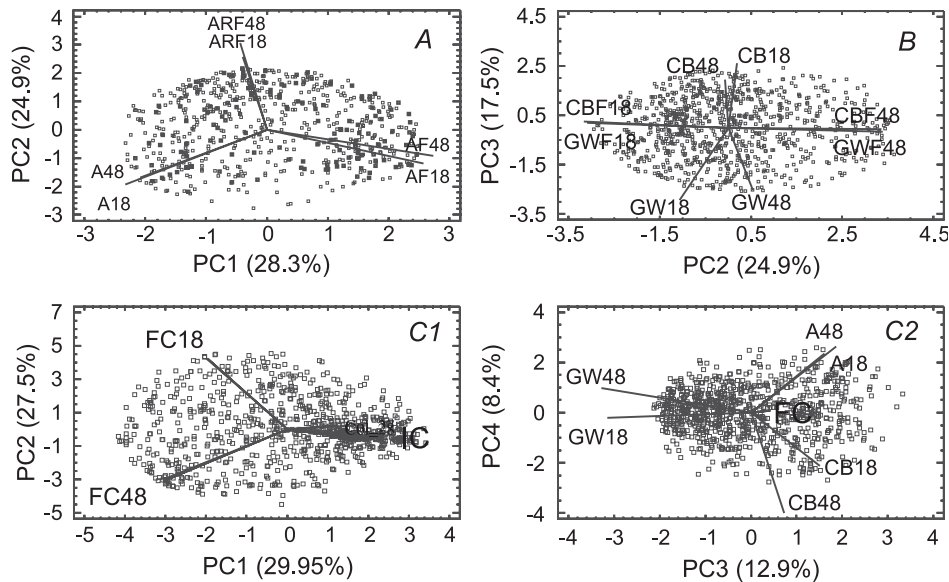


Fig. 2. Principal component analysis (PCA) of protein spot densities that were observed on 2D electropherograms obtained from planktonic and immobilized *P. aeruginosa* cells. Artificial (agar gel entrapment) and natural (biofilm formation on glass wool fibres or clay beads) immobilization procedures were tested as well as two durations of incubation (18 or 48 h). Incubation conditions and spot density values were the variables and the observations in PCA, respectively. To improve the separation of the observations by PCA, i.e. independently of the absolute amount of protein present in each detected spot, spot density values were standardized horizontally (i.e. converted to normal scores) in the data matrices. Biplots of scores and variable loadings are shown. The vectors represent loadings. Variables are indicated by abbreviations. Adapted from Vilain et al. (2004a, in press, c). (A) Artificial IC system. A data matrix of 923 rows (observations) $\times$ 6 columns (variables) was analysed. Biplot in PC1 $\times$ PC2 is shown. Variables (incubation conditions): F, free-cell cultures; AE, agar-entrapped cultures; ARF, agar-released, free-cell cultures. Numbers in variable abbreviations refer to the duration of incubation (18 or 48 h). (B) Natural IC systems. A data matrix of 914 rows $\times$ 8 columns was analysed. Biplot in PC2 $\times$ PC3 is shown. Variables: GWF, free-cell cultures in a bioreactor used for biofilm formation on glass wool; GW, biofilm cultures on glass wool; CBF, free-cell cultures in a bioreactor used for biofilm formation on clay beads; CB, biofilm cultures on clay beads. Numbers in variable abbreviations refer to the duration of incubation (18 or 48 h). (C1) and (C2) Artificial and natural IC systems. A data matrix of 933 rows $\times$ 12 columns was analysed. Biplots in (C1) PC1 $\times$ PC2 and (C2) PC3 $\times$ PC4 are shown. Variable abbreviations used in (C1): FC18, free-cell culture after incubation for 18 h (GWF18, CBF18 and AF18); FC48, free-cell culture after incubation for 48 h (GWF48, CBF48 and AF48); IC, immobilized-cell cultures (GW18, GW48, CB18, CB48, A18 and A48). Abbreviations used in (C2): FC, free-cell cultures (GWF18, GWF48, CBF18, CBF48, AF18 and AF48); others (immobilized-cell cultures), see above.

Table 8

Identification and function of proteins described as underproduced or overproduced in ICs compared to suspended counterparts

Protein function	Protein	Species/system	Level ^a	References
Membrane protein, transport	EF-Tu; lipoprotein Slp; OmpA; OmpX; TolC	<i>E. coli</i> /biofilm on hydrophobic glass beads	–	Otto and Silahvy, 2002
	Arginine/ornithine binding protein; probable binding protein component of ABC transporter; probable TonB-dependent receptor	<i>P. aeruginosa</i> entrapped in agar gel	–	Vilain et al., 2004b
	ABC transporter, PotF2; outer membrane lipoprotein NlpD	<i>P. putida</i> /biofilm on silicone tubing	–	Sauer and Camper, 2001
	Btub	<i>E. coli</i> /biofilm on hydrophobic glass beads	+	Otto and Silahvy, 2002
	Amino acid ABC transporter-binding protein YBEJ; D-ribose-binding periplasmic protein; D-galactose-binding protein	<i>E. coli</i> /biofilm on glass fibre filter	+	Trémoulet et al., 2002b
	Probable binding protein component of ABC transporter; Porin E	<i>P. aeruginosa</i> /biofilm on silicone tubing	+	Sauer et al., 2002
	Anaerobically induced OMP OprE precursor; molybdate-binding periplasmic protein ModA; binding protein of ABC phosphonate transporter	<i>P. aeruginosa</i> /biofilm on glass wool	+	Vilain et al., 2004c
	Anaerobically induced OMP OprE precursor; binding protein of ABC phosphonate transporter	<i>P. aeruginosa</i> /entrapment in agar gel	+	Vilain et al., 2004b
	Arginine deiminase ArcA; glutaminase asparaginase AnsB; ornithine carbamoyltransferase ArcB; serine-hydroxymethyltransferase GlyA3	<i>P. putida</i> /biofilm on silicone tubing	–	Sauer and Camper, 2001
	Dihydrolipoamide dehydrogenase 3	<i>P. aeruginosa</i> /biofilm on silicone tubing	–	Sauer et al., 2002
	Probable peroxidase; nitrogen regulatory protein P-II 2	<i>P. aeruginosa</i> /biofilm on clay beads	–	Vilain et al., 2004c
	Acetyl-CoA acetyltransferase; 3-hydroxyisobutyrate dehydrogenase; probable short-chain dehydrogenase; azurin precursor	<i>P. aeruginosa</i> /entrapment in agar gel	–	Vilain et al., 2004b
	Enolase; fructose biphosphate aldolase; glyceraldehyde-3-phosphate dehydrogenase; L-lactate dehydrogenase; 6-phosphofructokinase; pyruvate dehydrogenase; pyruvate kinase	<i>S. mutans</i> /biofilm on epon-hydroxyapatite (HA) rods	–	Svensäter et al., 2001
	Catabolic ornithine transcarbamylase cOTCase; L-lactate dehydrogenase (LctE); pyruvate dehydrogenase E1 component beta subunit (PdbB)	<i>Bacillus cereus</i> /biofilm on glass wool	+	Oosthuizen et al., 2002
Metabolism				

	Malate dehydrogenase; thiamine-phosphate pyrophosphate	<i>E. coli</i> /biofilm on glass fibre filter	+	Trémoulet et al., 2002b
	6-phosphofructokinase; pyruvate dehydrogenase	<i>L. monocytogenes</i> /biofilm on glass fibre filter	+	Trémoulet et al., 2002a
	Acylase, probable; adenylate kinase (purine biosynthesis); aminotransferase Class III, probable; arginine deiminase, AcrA; carbamate kinase; fumarate hydratase C1; glyceraldehyde-3-phosphate dehydrogenase; ketol-acid reductoisomerase; L-ornithine-5-monooxygenase (pyoverdine biosynthesis); ornithine carbamoyltransferase, catabolic, AcrB; succinate semialdehyde dehydrogenase; thioredoxine reductase (pyrimidine biosynthesis; UTP-glucose-1-phosphate uridylyltransferase	<i>P. aeruginosa</i> /biofilm on silicone tubing	+	Sauer et al., 2002
	Probable iron–sulfur protein; orotate phosphoribosyltransferase	<i>P. aeruginosa</i> /biofilm on clay beads	+	Vilain et al., 2004c
	Phenylalanine-4-hydroxylase; Lipamide dehydrogenase-glc; acetyl-CoA acetyltransferase; NADH dehydrogenase I chain M; 2-keto-3-deoxy-6-phosphogluconate aldolase; leucine dehydrogenase; probable short-chain dehydrogenase; acetolactate synthase isozyme III small subunit; orotate phosphoribosyltransferase;	<i>P. aeruginosa</i> /biofilm on glass wool	+	Vilain et al., 2004c
	phosphoribosylaminoimidazole carboxylase			
	Phospho-2-dehydro-3-deoxyheptonate chain	<i>S. mutans</i> /biofilm on HA rods	+	Svensäter et al., 2001
DNA replication	ATP-dependent DNA helicase RECG;	<i>S. mutans</i> /biofilm on HA rods	–	Svensäter et al., 2001
	triosephosphate isomerase			
Transcription–translation–elongation	Elongation factor Tu; elongation factor Ts; ribosome recycling factor	<i>S. mutans</i> /biofilm on HA rods	+	Svensäter et al., 2001
	Probable ribosomal protein L25	<i>P. aeruginosa</i> entrapped in agar gel	–	Vilain et al., 2004b
	50S ribosomal protein L10	<i>P. aeruginosa</i> /biofilm on clay beads	+	Vilain et al., 2004c
	RsmA, regulator of secondary metabolites; ribosome recycling factor; transcription elongation factor GreA	<i>P. aeruginosa</i> /biofilm on glass wool	+	Vilain et al., 2004c

(continued on next page)

Table 8 (continued)

Protein function	Protein	Species/system	Level ^a	References
Motility	Twitching motility protein PilH	<i>P. aeruginosa</i> /biofilm on glass wool	+	Vilain et al., 2004c
Adaptation,	Bacterioferritin comigratory protein; pyocin S2	<i>P. aeruginosa</i> /biofilm on clay beads	–	Vilain et al., 2004c
Protection,	immunity protein; Heat-shock protein IbpA			
Protein folding	Thiol–disulfide interchange protein DsbA	<i>P. aeruginosa</i> /biofilm on glass wool	–	Vilain et al., 2004c
	Bacterioferritin comigratory protein; heat-shock protein IbpA	<i>P. aeruginosa</i> entrapped in agar gel	–	Vilain et al., 2004b
	60 kDa chaperonin	<i>S. mutans</i> /biofilm on HA rods	–	Svensäter et al., 2001
	YhbH light-repressed protein A	<i>B. cereus</i> /biofilm on glass wool	+	Oosthuizen et al., 2002
	DNA-binding protein Dps; DNA-binding protein H-NS	<i>E. coli</i> /biofilm on glass fibre filter	+	Trémoulet et al., 2002b
	30S ribosomal protein S2 (rpsB); superoxide dismutase; YvyD	<i>L. monocytogenes</i> /biofilm on glass fibre filter	+	Trémoulet et al., 2002a
	Probable cold-shock protein	<i>P. aeruginosa</i> /biofilm on clay beads	+	Vilain et al., 2004c
	Alkyl hydroxyperoxide reductase subunit C; helix-destabilizing protein of bacteriophage Pfl; probable ribosomal protein L25; superoxide dismutase	<i>P. aeruginosa</i> /biofilm on silicone tubing	+	Sauer et al., 2002
	Pyocin S2 immunity protein; probable cold-shock protein; heat-shock protein IbpA	<i>P. aeruginosa</i> /biofilm on glass wool	+	Vilain et al., 2004c
	Pyocin S2 immunity protein	<i>P. aeruginosa</i> /entrapment in agar gel	+	Vilain et al., 2004b
	DnaK; GrpE protein; Trigger factor PPIASE	<i>S. mutans</i> /biofilm on HA rods	+	Svensäter et al., 2001
Nucleotide biosynthesis	Formate tetrahydrofolate ligase	<i>S. mutans</i> /biofilm on HA rods	–	Svensäter et al., 2001

^a (–) Underproduced; (+) overproduced.

cells cultured as suspensions or in the immobilized state for 18 or 48 h. PCA of proteomic data from agar gel entrapped (A), free (suspended) (AF) and agar-released, free (ARF) organisms (Vilain et al., 2004b) extracted three components (with eigenvalues higher than 1) together accounting for 71.6% of the variability in the data. The diagram of scores and variable loadings in PC1×PC2 (Fig. 2A) allowed to discriminate between the three tested culture modes, independently of the duration of incubation. Principal component 1 (PC1) opposed A and AF cultures, with a low contribution of ARF cultures to PC1. Inversely, the contribution of ARF cultures to PC2 was high, opposing those of A and AF cultures. Component 3 was related to the duration of incubation. The same statistical analysis was performed on protein maps from bacteria cultured as biofilms on two different supports, i.e. glass wool fibres (GW) and clay beads CB) (Vilain et al., 2004a). PCA again extracted three components explaining 78.4% of the variability in the data. Component 1 opposed free-cell cultures to biofilm ones. Component 2 was related essentially to free-cell cultures, discriminating between the two tested incubation times. Component 3 opposed the two modes of biofilm growth (Fig. 2B). Therefore, the bacterial mode of growth, i.e. suspended or attached, was the main parameter controlling spot intensity variations in protein maps. The duration of incubation, more significant for free cells than for biofilm bacteria, and the nature of the substratum used for biofilm development also contributed to the observed modifications in 2D electropherograms. This statistical demonstration of the influence exerted by the substratum nature on protein expression in biofilm cells has been confirmed experimentally by recent results showing that the resistance of attached bacteria to antimicrobials was dependent on the nature of the biofilm support (Deng et al., 2004). Finally, PCA was extended to the whole set of proteomic data (Vilain et al., 2004c), i.e. protein maps from biofilm and gel-entrapped bacteria (Fig. 2C). It extracted four components, accounting together for 78.75% of the variability. PC1 opposed the two modes of growth (planktonic and immobilized), while IC growth conditions showed negligible weight on PC2 that discriminated between the incubation times of free cell cultures (Fig. 2C1). The incubation conditions of ICs, including the immobilization procedure (entrapment vs. attachment) and the nature of the biofilm substratum, were fairly separated in PC3×PC4 (Fig. 2C2). These comparative analyses of bacterial protein patterns in suspended and immobilized organisms demonstrate that the protein contents of ICs *sensu lato* (i.e. naturally attached or artificially entrapped cells) can be statistically differentiated from those of free, suspended counterparts. The two tested immobilization processes and IC culture modes show evident differences, for instance the absence in gel-entrapped cultures of the initial adhesion step and early development stage inherent to biofilms—periods during which changes in gene expression and protein patterns actually occur in attached organisms (Sauer and Camper, 2001). The statistical analogy between the protein maps of organisms belonging to these quite different IC systems as compared to free-cell proteomes reinforces the topical hypothesis that bacteria in the immobilized state display a specific physiological behaviour (Drenkert and Ausubel, 2002) and opposes Hancock's assertion (2001). The results of PCA also cast doubts on the existence of a unique IC phenotype (Davies, 2003), however, since the nature of the substratum used for biofilm development was shown to contribute to the observed modifications in 2D electropherograms.

The statistical analysis of proteome changes induced by immobilization obviously did not distinguish between trivial and key polypeptides whose variations in the expression level are likely to influence IC physiology: a question that arises is the identification of biofilm-specific expression levels. A number of proteins whose amount varied in ICs compared to suspended counterparts have been identified by more “conventional” exploitation of 2D-electropherograms (Table 8). These proteins can be divided into three main classes. The first class is composed of membrane proteins. Membrane proteins have been reported to have a substantial influence on attachment and may also play a role in early biofilm development (Schembri and Klemm, 2001; Coquet et al., 2002; Otto and Silahvy, 2002). They are implied in multidrug resistance pumps of gram-negative bacteria (Aires et al., 1999; Köhler et al., 1999) and their over/underproduction by ICs may therefore be implied in IC resistance to antibiotics. The second class includes proteins linked to metabolic processes, such as amino acid and cofactor biosyntheses, showing not surprisingly that central metabolism is affected by the sessile mode of growth. The last class includes proteins involved in adaptation and protection. While no clear expression tendency of proteins belonging to the first two classes can be discerned (some are up-regulated while others are down-regulated), most adaptation proteins are accumulated by biofilm bacteria. This general stress response initiated by growth within a biofilm might explain the resistance of sessile cells to environmental stresses (Brown and Barker, 1999). Some contradictions in the expression level of some proteins can be observed. For example, the enzymes L-lactate dehydrogenase, ornithine carbamoyltransferase, 6-phosphofructokinase and pyruvate dehydrogenase have been described as up- and down-regulated. Furthermore, a great number of proteins involved in the biofilm phenotype remain with an unknown function.

Identifying target peptides among this wealth of proteins differentially expressed by ICs as compared to free counterparts seems a difficult challenge. It may also be difficult (and sometimes dangerous) to advance a specific role for a given over/underexpressed protein in the biofilm phenotype—though interpretations are possible in some limited cases. Therefore, the best strategy to identify “biofilm” proteins is probably a mutagenesis approach based on proteomic data.

5. Conclusion

Viable IC technologies have developed rapidly over the last 30 years. A lot of practical applications of IC systems have been proposed during this period and the field is always topical. A very large majority of these applications remain at the laboratory scale, however. For a long time, process implementation has monopolized the research efforts that in return deserted more basic studies on IC behaviour. A typical illustration of this paradoxical evolution is given by the early success of IC cultures concerning the alcoholic fermentation and the biodegradation of toxic compounds, while the cellular origins of the high resistance of ICs to adverse environmental conditions such as the exposure to antimicrobial agents have been only recently investigated and remain to be fully understood. Faced with that situation, the emergence of proteomics as a powerful tool to compare the global regulation patterns of gene expression in free and immobilized

microbial cells opens promising avenues to the study of IC physiology. Recent developments in proteomics of ICs (together with genomic and transcriptomic approaches) already offer original information on the physiological behaviour of ICs: in particular, they show that bacteria growing in the immobilized state are physiologically different from free-living organisms. The alliance of the proteomic approach with classical tools of molecular biology will, in the near future, probably allow us to identify key proteins whose over/underexpression exerts deciding influence on IC physiology.

Will these in-depth investigations of the physiological behaviour of microorganisms living in the immobilized state be useful to strengthen the practical potentialities of IC technology, improving the efficiency of biotechnological processes based on ICs? An exhaustive answer to this question is uneasy at the present time as concerns bioproduction and biodegradation processes. Such studies will help to balance the practical, historically claimed advantages of ICs against the boundaries of the technology incidental to the peculiar physiology of ICs. For instance, a better knowledge of stress and starvation phenomena endured by ICs, of the metabolic pathways affected by immobilization will likely allow to discriminate between unrealistic and sound application fields of the technology (e.g. biodegradation of recalcitrant compounds and the production of secondary metabolites). The answer is much easier concerning biofilms implied in infections and industrial biofouling since proteomic studies will probably lead to the identification of targets proteins to fight against these undesirable IC systems—the improvement of weapons against biofilm-based infections and biofouling being an ambitious goal that is offered to medical and environmental microbiologists.

References

- Abdel-Naby MA, Reyad RM, Abdel-Fattah AF. Biosynthesis of cyclodextrin glucosyltransferase by immobilized *Bacillus amyloliquefaciens* in batch and continuous cultures. *Biochem Eng J* 2000;5:1–9.
- Aires JR, Köhler T, Nikaido H, Plésiat P. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 1999;43:2624–8.
- Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* 2000;44:1818–24.
- Anderl JN, Zahller J, Roe F, Stewart PS. Role of nutrient limitation and stationary-phase existence in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* 2003;47:1251–6.
- Angelova MB, Pashova SB, Slokoska LS. Comparison of antioxidant enzyme biosynthesis by free and immobilized *Aspergillus niger* cells. *Enzyme Microb Technol* 2000;26:544–9.
- Annadurai G, Rajesh Babu S, Mahesh KPO, Murugesan T. Adsorption and bio degradation of phenol by chitosan-immobilized *Pseudomonas putida* (NICM 2174). *Bioprocess Eng* 2000;22:493–501.
- Anselmo AM, Novais JM. Biological treatment of phenolic wastes: comparison between free and immobilized cell systems. *Biotechnol Lett* 1992;14:239–44.
- Ashby MJ, Neale JE, Knott SJ, Critchley IA. Effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. *J Antimicrob Chemother* 1994;33:443–52.
- Azanta Teruel ML, Gontier E, Biennaimé C, Nava Saucedo JE, Barbotin J-N. Response surface analysis of chlortetracycline and tetracycline production with κ -carrageenan immobilized *Streptomyces aureofaciens*. *Enzyme Microb Technol* 1997;21:314–20.
- Bergamini TM, McCurry TM, Bernard JD, Hoeg KL, Corpus RA, Peyton JC, et al. Antibiotic efficacy against *Staphylococcus epidermidis* adherent to vascular grafts. *J Surg Res* 1996;60:3–6.

- Bickerstaff GF, editor. Immobilization of Enzymes and Cells. Totowa (NJ): Humana Press; 1997.
- Brányik T, Kuncová G, Páca J. The use of silica gel prepared by sol–gel method and polyurethane foam as microbial carriers in the continuous degradation of phenol. Appl Microbiol Biotechnol 2000;54:168–72.
- Brown MRW, Barker J. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. Trends Microbiol 1999;7:46–50.
- Cash P. Characterisation of bacterial proteomes by two-dimensional electrophoresis. Anal Chim Acta 1998;372:121–45.
- Cash P. Proteomics of bacterial pathogens. Adv Biochem Eng Biotechnol 2003;83:93–115.
- Cassidy MB, Shaw KW, Lee H, Trevors JT. Enhanced mineralization of pentachlorophenol by κ -carrageenan-encapsulated *Pseudomonas* sp UG30. Appl Microbiol Biotechnol 1997;47:108–13.
- Chen K-C, Huang C-T. Effects of the growth of *Trichosporon cutaneum* in calcium alginate gel beads upon bead structure and oxygen transfer characteristics. Enzyme Microb Technol 1988;10:284–92.
- Chuard C, Vaudaux P, Waldvogel FA, Lew DP. Susceptibility of growing on fibronectin-coated surfaces to bactericidal antibiotics. Antimicrob Agents Chemother 1993;37:625–32.
- Chun G-T, Agathos SN. Comparative studies of physiological and environmental effects on the production of cyclosporin A in suspended and immobilized cells of *Tolypocladium inflatum*. Biotechnol Bioeng 1991;37:256–65.
- Coquet L, Junter G-A, Jouenne T. Resistance of artificial biofilms of *Pseudomonas aeruginosa* to imipenem and tobramycin. J Antimicrob Chemother 1998;42:755–60.
- Coquet L, Cosette P, Quillet L, Petit F, Junter G-A, Jouenne T. Occurrence and phenotypic characterization of *Yersinia ruckeri* strains with biofilm-forming capacity in a rainbow trout farm. Appl Environ Microbiol 2002;68:470–5.
- Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999;284:1318–22.
- Davies D. Understanding biofilm resistance to antimicrobial agents. Nat Rev 2003;2:114–22.
- Demuyakor B, Ohta T. Promotive action of ceramics on yeast ethanol production, and its relationship to pH, glycerol and alcohol dehydrogenase activity. Appl Microbiol Biotechnol 1992;36:717–21.
- Deng DM, Buijs MJ, ten Cate JM. The effects of substratum on the pH response of *Streptococcus mutans* biofilms and on the susceptibility to 0.2% chlorhexidine. Eur J Oral Sci 2004;112:42–7.
- Dervakos GA, Webb C. On the merits of viable-cell immobilisation. Biotechnol Adv 1991;9:559–612.
- Doran PM, Bailey JE. Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin. Biotechnol Bioeng 1986;28:73–87.
- Drenkert E, Ausubel FM. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 2002;416:740–3.
- Durham DR, Marshall LC, Miller JG, Chmurny AB. Characterization of inorganic biocarriers that moderate system upsets during fixed-film biotreatment processes. Appl Environ Microbiol 1994;60:3329–35.
- Dykes GA, Sampathkumar B, Korber DR. Planktonic or biofilm growth affects survival, hydrophobicity and protein expression patterns of a pathogenic *Campylobacter jejuni* strain. Int J Food Microbiol 2003;89:1–10.
- Fang HHP, Zhou GM. Denitrification of phenolic wastewater by immobilized sludge. Environ Technol 1997;18:827–34.
- Farid MA, Abu-Shady MR, El-Diwany AI, El-Enshasy HA. Production of rifamycin B and SV by free and immobilized cells of *Amiclatopsis mediterranei*. Acta Biotechnol 1995;15:241–8.
- Freeman A, Lilly MD. Effect of processing parameters on the feasibility and operational stability of immobilized viable microbial cells. Enzyme Microb Technol 1998;23:335–45.
- Galazzo JL, Bailey JE. In vivo nuclear magnetic resonance analysis of immobilization effects on glucose metabolism of yeast *Saccharomyces cerevisiae*. Biotechnol Bioeng 1989;33:1283–9.
- Galazzo JL, Bailey JE. Growing *Saccharomyces cerevisiae* in calcium-alginate beads induces cell alterations which accelerate glucose conversion to ethanol. Biotechnol Bioeng 1990;36:417–26.
- Gerbsch N, Buchholz R. New processes and actual trends in biotechnology. FEMS Microbiol Rev 1995;16:259–69.
- Gestrelus S. Immobilized nonviable cells for use of a single or a few enzyme steps. In: Mattiasson B, editor. Immobilized Cells and Organelles, vol. 2. Boca Raton (FL): CRC Press; 1983. p. 1–22.
- Ghigo J-M. Are there biofilm-specific physiological pathways beyond a reasonable doubt? Res Microbiol 2003;154:1–8.

- González G, Herrera G, García Ma T., Peña M. Biodegradation of phenol in a continuous process: comparative study of stirred tank and fluidized-bed bioreactors. *Bioresour Technol* 2001a;76:245–51.
- González G, Herrera G, García Ma T., Peña M. Biodegradation of phenolic industrial wastewater in a fluidized bed bioreactor with immobilized cells of *Pseudomonas putida*. *Bioresour Technol* 2001b;80:137–42.
- Guina T, Wu M, Miller SI, Purvine SO, Yi EC, Eng J, et al. Proteomic analysis of *Pseudomonas aeruginosa* grown under magnesium limitation. *J Am Soc Mass Spectrom* 2003;14:742–51.
- Habash M, Reid G. Microbial biofilms: their development and significance for medical device-related infections. *J Clin Pharmacol* 1999;39:887–98.
- Hancock REW. A brief on bacterial biofilms. *Nat Genet* 2001;29:360.
- Hecker M, Engelmann S, Cordwell SJ. Proteomics of *Staphylococcus aureus*—current state and future challenges. *J Chromatogr B, Biomed Sci Appl* 2003;787:179–95.
- Hilge-Rotmann B, Rehm H-J. Relationship between fermentation capability and fatty acid composition of free and immobilized *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1991;34:502–8.
- Høiby N. Understanding bacterial biofilms in patients with cystic fibrosis: current and innovative approaches to potential therapies. *J Cyst Fibros* 2002;1:249–54.
- Hu Z-C, Korus RA, Levinson WE, Crawford RL. Adsorption and biodegradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium*. *Environ Sci Technol* 1994;28:491–6.
- Jirku V. Whole cell immobilization as a means of enhancing ethanol tolerance. *J Ind Microbiol Biotech* 1999;22:147–51.
- Jouenne T, Tresse O, Junter G-A. Agar-entrapped bacteria as an in vitro model of biofilms and their susceptibility to antibiotics. *FEMS Microbiol Lett* 1994;119:237–42.
- Junter G-A, Coquet L, Vilain S, Jouenne T. Immobilized-cell physiology: current data and the potentialities of proteomics. *Enzyme Microb Technol* 2002a;31:201–12.
- Junter G-A, Jouenne T, Vilain S. Biological treatment of water using immobilized-cell systems: III Phenolic compounds and other monoaromatics: Part 1. *Chim Oggi* 2002b;20(6):57–62.
- Kalya AV, Ahearn DG. Increased resistance to antifungal antibiotics of *Candida* spp adhered to silicone. *J Ind Microbiol* 1995;14:451–5.
- Karel SF, Libicki SB, Robertson CR. The immobilization of whole cells: engineering principles. *Chem Eng Sci* 1985;40:1321–54.
- Karel SF, Salmon PM, Stewart PS, Robertson CR. Reaction and diffusion in immobilized cells: fact and fantasy. In: de Bont JAM, Visser J, Mattiasson B, Tramper J, editors. *Physiology of immobilized cells*. Amsterdam: Elsevier; 1990. p. 115–26.
- Klingeberg M, Vorlop KD, Antrakinian C. Immobilization of anaerobic thermophilic bacteria for the production of cell-free thermostable α -amylases and pullulanases. *Appl Microbiol Biotechnol* 1990;33:494–500.
- Köhler T, Michea-Hamzehpour M, Epp SF, Pechere JC. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. *Antimicrob Agents Chemother* 1999;43:424–7.
- König C, Schwank S, Blaser J. Factors compromising antibiotic activity against biofilms of *Staphylococcus epidermidis*. *Eur J Clin Microbiol Infect Dis* 2001;20:20–6.
- Kumon H, Ono N, Iida M, Nickel JC. Combination effect of fosfomycin and ofloxacin against *Pseudomonas aeruginosa* growing in a biofilm. *Antimicrob Agents Chemother* 1995;39:1038–44.
- Larsen T. Susceptibility of *Porphyromonas gingivalis* in biofilms to amoxicillin, doxycycline and metronidazole. *Oral Microbiol Immunol* 2002;17:267–71.
- Len ACL, Cordwell SJ, Harty DWS, Jacques NA. Cellular and extracellular proteome analysis of *Streptococcus mutans* grown in a chemostat. *Proteomics* 2003;3:627–46.
- Liao X, Ying T, Wang H, Wang J, Shi Z, Feng E, et al. A two-dimensional proteome map of *Shigella flexneri*. *Electrophoresis* 2003;24:2864–82.
- Lohmeier-Vogel EM, McIntyre DD, Vogel HJ. Phosphorus-31 and Carbon-13 nuclear magnetic resonance studies of glucose and xylose metabolism in cell suspensions and agarose-immobilized cultures of *Pichia stipitis* and *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 1996;62:2832–8.
- Longo MA, Novella IS, García LA, Diaz M. Comparison of *Bacillus subtilis* and *Serratia marcescens* as protease producers under different operating conditions. *J Biosci Bioeng* 1999;88:35–40.
- Loo CY, Corliss DA, Ganeshkumar N. *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *J Bacteriol* 2000;182:1374–82.

- Luke AK, Burton SG. A novel application for *Neurospora crassa*: progress from batch culture to a membrane bioreactor for the bioremediation of phenols. *Enzyme Microb Technol* 2001;29:348–56.
- Lyngberg OK, Thiagarajan V, Stemke DJ, Schottel JL, Scriven LE, Flickinger MC. A patch coating method for preparing biocatalytic films of *Escherichia coli*. *Biotechnol Bioeng* 1999;62:44–55.
- Mah T-FC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 2001;9:34–9.
- Mattiasson B, editor. Immobilized cells and organelles, vols. 1 and 2. Boca Raton (FL): CRC Press; 1983a.
- Mattiasson B. Immobilized viable cells. Mattiasson B, editor. Immobilized cells and organelles, vol. 2. Boca Raton (FL): CRC Press; 1983b. p. 23–40.
- Mordocco A, Kuek C, Jenkins R. Continuous degradation of phenol at low concentration using immobilized *Pseudomonas putida*. *Enzyme Microb Technol* 1999;25:530–6.
- Muyima NYO, Cloete TE. Growth and phosphate uptake of immobilized *Acinetobacter* cells suspended in activated sludge mixed liquor. *Water Res* 1995;29:2461–6.
- Nawaz MS, Franklin W, Cerniglia CE. Degradation of acrylamide by immobilized cells of a *Pseudomonas* sp and *Xanthomonas maltophilia*. *Can J Microbiol* 1993;39:207–12.
- Nickel JC, Ruseska I, Wright JB, Costerton JW. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob Agents Chemother* 1985;27:619–24.
- Nicolella C, van Loosdrecht MCM, Heijnen SJ. Particle-based biofilm reactor technology. *Trends Biotechnol* 2000;18:312–20.
- Nkhalambayausi-Chirwa EM, Wang Y-T. Simultaneous chromium (VI) reduction and phenol degradation in a fixed-film coculture bioreactor: reactor performance. *Water Res* 2001;35:1921–32.
- Norton S, D'Amore T. Physiological effects of yeast cell immobilization: applications for brewing. *Enzyme Microb Technol* 1994;16:365–75.
- O'Connor CD, Adams P, Alefounder P, Farris M, Kinsella N, Li Y, et al. The analysis of microbial proteomes: strategies and data exploitation. *Electrophoresis* 2000;21:1178–86.
- Oosthuizen MC, Steyn B, Theron J, Cosette P, Lindsay D, von Holy A, et al. Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. *Appl Environ Microbiol* 2002;68:2770–80.
- O'Reilly KT, Crawford RL. Degradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium* cells. *Appl Environ Microbiol* 1989;55:2113–8.
- Otto K, Silahvy TJ. Surface sensing and adhesion of *Escherichia coli* under control of the Cpx signalling pathway. *Proc Natl Acad Sci U S A* 2002;99:2287–92.
- Otto K, Norbeck J, Larsson T, Karlsson K-L, Hermansson M. Adhesion of type 1-fimbriated *Escherichia coli* to abiotic surfaces leads to altered composition of outer membrane proteins. *J Bacteriol* 2001;183:2445–53.
- Pai S-L, Hsu Y-L, Chong N-M, Sheu CS, Chen C-H. Continuous degradation of phenol by *Rhodococcus* sp immobilized on granular activated carbon and in calcium alginate. *Bioresour Technol* 1995;51:37–42.
- Parascandola P, de Alteriis E, Sentandreu R, Zueco J. Immobilization and ethanol stress induce the same molecular response at the level of the cell wall in growing yeast. *FEMS Microbiol Lett* 1997;150:121–6.
- Pashova S, Slokoska L, Sheremetska P, Krumova E, Vasileva L, Angelova M. Physiological aspects of immobilised *Aspergillus niger* cells producing polymethylgalacturonase. *Proc Biochem* 1999;35:15–9.
- Perrot F, Hébraud M, Charlonet R, Junter G-A, Jouenne T. Protein patterns of gel-entrapped *Escherichia coli* differ from those of free-floating organisms. *Electrophoresis* 2000;21:645–53.
- Pilkington PH, Margaritis AH, Mensour NA. Mass transfer characteristics of immobilized cells used in fermentation processes. *Crit Rev Biotechnol* 1998;18:237–55.
- Preston CAK, Khoury AE, Reid G, Bruce AW, Costerton JW. *Pseudomonas aeruginosa* biofilms are more susceptible to ciprofloxacin than to tobramycin. *Int J Antimicrob Agents* 1996;7:251–6.
- Prigent-Combaret C, Vidal O, Dorel C, Lejeune P. Abiotic surface-sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *J Bacteriol* 1999;181:5993–6002.
- Radovich JM. Mass transfer effects in fermentations using immobilized whole cells. *Enzyme Microb Technol* 1985;7:2–10.
- Ramage G, Tunney MM, Patrick S, Gorman SP, Nixon JR. Formation of *Propionibacterium acnes* biofilms on orthopaedic biomaterials and their susceptibility to antimicrobials. *Biomaterials* 2003;24:3221–7.
- Riley MR, Muzzio FJ, Reyes SC. Experimental and modeling studies of diffusion in immobilized cell systems. *Appl Biochem Biotechnol* 1999;80:151–88.

- Rosevear A, Kennedy JF, Cabral JMS. Immobilised enzymes and cells. Bristol: Adam Hilger; 1987.
- Santos-Rosa F, Galván F, Vega JM. Biological viability of *Chlamydomonas reinhardtii* cells entrapped in alginate beads for ammonium photoproduction. *J Biotechnol* 1989;9:209–20.
- Sauer K, Camper AK. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol* 2001;183:6579–89.
- Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 2002;184:1140–54.
- Schembri MA, Klemm P. Coordinate gene regulation by fimbriae-induced signal transduction. *EMBO J* 2001;20:3074–81.
- Schembri MA, Kjærsgaard K, Klemm P. Global gene expression in *Escherichia coli* biofilms. *Mol Microbiol* 2003;48:253–67.
- Scott RI, Sladen S, Maidment M, Tashid T, Pratsis C, Perry D. The effects of oxygen on β -lactam biosynthesis by alginate-entrapped *Streptomyces clavuligerus*. *J Chem Technol Biotechnol* 1988;41:145–54.
- Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 2000;407:762–4.
- Sonomoto K, Chinachoti N, Endo N, Ishizaki A. Biosynthetic production of nisin Z by immobilized *Lactococcus lactis* IO-1. *J Mol Catal, B Enzym* 2000;10:325–34.
- Stickler DJ, McLean RJC. Biomaterials associated infections: the scale of the problem. *Cells Mater* 1995;5:167–82.
- Stone G, Wood P, Dixon L, Keyhan M, Matin A. Tetracycline rapidly reaches all the constituent cells of uropathogenic *Escherichia coli* biofilms. *Antimicrob Agents Chemother* 2002;46:2458–61.
- Suzuki T, Yamaguchi T, Ishida M. Immobilization of *Prototheca zopfii* in calcium-alginate beads for the degradation of hydrocarbons. *Process Biochem* 1998;33:541–6.
- Svensäter G, Welin J, Wilkins JC, Beighton D, Hamilton IR. Protein expression by planktonic and biofilm cells of *Streptococcus mutans*. *FEMS Microbiol Lett* 2001;205:139–46.
- Taipa MA, Cabral JMS, Santos H. Comparison of glucose fermentation by suspended and gel-entrapped yeast cells: an in vivo nuclear magnetic resonance study. *Biotechnol Bioeng* 1993;41:647–53.
- Tampion J, Tampion MD. Immobilized cells: principles and applications. Cambridge Studies in Biotechnology, vol. 5. Cambridge: Cambridge University Press; 1987.
- Tanaka G, Shigeta M, Komatsuzawa H, Sugai M, Suganaka H, Usui T. Effect of the growth rate of *Pseudomonas aeruginosa* biofilms on the susceptibility to antimicrobial agents: beta-lactams and fluoroquinolones. *Chemotherapy* 1999;45:28–36.
- Teng R, Dick T. Isoniazid resistance of exponentially growing *Mycobacterium smegmatis* biofilm culture. *FEMS Microbiol Lett* 2003;227:171–4.
- Trauth E, Lemaître J-P, Rojas C, Diviès C, Cachon R. Resistance of immobilized lactic acid bacteria to the inhibitory effect of quaternary ammonium sanitizers. *Lebensm-Wiss Technol* 2001;34:239–43.
- Trémoulet F, Duché O, Namane A, Martinie B, Labadie J-C, European Listeria Genome Consortium. Comparison of protein patterns of *Listeria monocytogenes* grown in biofilm or in planktonic mode by proteomic analysis. *FEMS Microbiol Lett* 2002a;210:25–31.
- Trémoulet F, Duché O, Namane A, Martinie B, Labadie J-C. A proteomic study of *Escherichia coli* O157:H7 NCTC 12900 cultivated in biofilm or in planktonic growth mode. *FEMS Microbiol Lett* 2002b;215:7–14.
- Tresse O, Jouenne T, Junter G-A. The role of oxygen limitation in the resistance of agar-entrapped, sessile-like *Escherichia coli* to aminoglycoside and β -lactam antibiotics. *J Antimicrob Chemother* 1995;36:521–6.
- VanBogelen RA. Probing the molecular physiology of the microbial organism, *Escherichia coli* using proteomics. *Adv Biochem Eng Biotechnol* 2003;83:27–55.
- VanBogelen RA, Schiller EE, Thomas JD, Neidhardt FC. Diagnosis of cellular states of microbial organisms using proteomics. *Electrophoresis* 1999;20:2149–59.
- Van Iersel MFM, Brouwer-Post E, Rombouts FM, Abee T. Influence of yeast immobilization on fermentation and aldehyde reduction during the production of alcohol-free beer. *Enzyme Microb Technol* 2000;26:602–7.
- Veliky IA, McLean RJC. Immobilized biosystems: theory and practical applications. Dordrecht: Kluwer Academic Publishers; 1993.
- Vieth WR, Venkatsubramanian K. Immobilized microbial cells in complex biocatalysis. In: Venkatsubramanian K, editor. Immobilized microbial cells. ACS Symposium Series, vol. 106. Washington (DC): American Chemical Society; 1979. p. 1–11.

- Vilain S, Cosette P, Zimmerlin I, Dupont J-P, Junter G-A, Jouenne T. Biofilm proteome: homogeneity or versatility? *J Proteome Res* 2004a;3:132–6.
- Vilain S, Cosette P, Hubert M, Lange C, Junter G-A, Jouenne T. A proteomic analysis of agar gel entrapped *Pseudomonas aeruginosa*. *Proteomics* 2004b;4:1996–2004.
- Vilain S, Cosette P, Hubert M, Lange C, Junter G-A, Jouenne T. Comparative proteomic analysis of planktonic and immobilized *Pseudomonas aeruginosa* cells: a multivariate statistical approach. *Anal Biochem* 2004c;329:120–30.
- Vuong C, Otto M. *Staphylococcus epidermidis* infections. *Microb Infect* 2002;4:481–9.
- Wagner MA, Eschenbrenner M, Horn TA, Kraycer JA, Mujer CV, Hagius S, et al. Global analysis of the *Brucella melitensis* proteome: identification of proteins expressed in laboratory-grown culture. *Proteomics* 2002;2:1047–60.
- Walsh PK, Malone DM. Cell growth patterns in immobilization matrices. *Biotechnol Adv* 1995;13:13–43.
- Washburn MP, Yates III JR. Analysis of the microbial proteome. *Curr Opin Microbiol* 2000;3:292–7.
- Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 2001;413:860–4.
- Wijffels RH, editor. Immobilized cells. Heidelberg: Springer Verlag; 2001.
- Willaert R, Baron G. Growth kinetics of gel-immobilized yeast cells studied by on-line microscopy. *Appl Microbiol Biotechnol* 1993;39:347–52.
- Williams I, Venables WA, Lloyd D, Paul F, Critchley I. The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus*. *Microbiology (UK)* 1997;143:2407–13.
- Wright TL, Ellen RP, Lacroix JM, Sinnadurai S, Mittelman MW. Effects of metronidazole on *Porphyromonas gingivalis* biofilms. *J Periodontal Res* 1997;32:473–7.
- Zache G, Rehm H-J. Degradation of phenol by a coimmobilized entrapped mixed culture. *Appl Microbiol Biotechnol* 1989;30:426–32.

Standard Technical Procedures for Microencapsulation of Human Islets for Graft into Nonimmunosuppressed Patients With Type 1 Diabetes Mellitus

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ABSTRACT

To comply with regulatory restrictions, with regard to graft of human islets immunoprotected within artificial microcapsules, into patients with type 1 diabetes mellitus (T1DM) with no recipient immunosuppression, we have prepared standard protocols on: (1) sodium alginate purification (clinical grade) for microcapsule fabrication; (2) preparation of biocompatible and permselective microcapsules containing human islets; and (3) minimally invasive techniques for grafting of the encapsulated human islets into the recipients' peritoneal cavity. As to no. 1, starting from pharmaceutical grade, raw sodium alginate powder, we prepared a pyrogen- and endotoxin-free 1.6% alginate solution by means of dialysis, multiple filtrations, and dilution/osmolality adjustments. As to no. 2, we have selected human islet preparations associated with >80% purity/viability, which underwent careful functional quality control testing prior to encapsulation; namely, most capsules contained one islet. As for no. 3, we have devised a simple intraperitoneal injection method under abdominal echography guidance with only local anesthesia to deposit the encapsulated islets in saline within the peritoneal leaflets. These technical protocols were officially approved by the Italian Ministry of Health which has released permission to conduct a phase I, closed human trial in 10 patients using encapsulated human islet grafts into nonimmunosuppressed patients with T1DM.

TWO MAJOR LIMITS restrain widespread application of islet transplantation as a potential cure for type 1 diabetes mellitus (T1DM), namely, need for lifelong general immunosuppression of the recipients, and gap between availability and demand for human islet tissue. A potential exit strategy for both issues could be to avoid recipient immunosuppression by protecting the islet grafts within selectively permeable microcapsules made of Na alginate (AG) and poly-L-ornithine (PLO), as previously described by us.¹ This approach may eventually allow access to nonhuman tissue (ie, porcine) as a resource for donor islets. While multiple experimental studies^{2,3} have clearly indicated that microencapsulation is a safe and effective procedure, provided that appropriate reagents and technical procedures are available, no guidelines have ever been communicated for human application. We undertook the task of preparing standard operative procedures for human islet cell microencapsulation and their transplantation into patients with T1DM, after audits and discussions with the Italian Ministry of Health. Care was taken to design precise

protocols that complied with regulatory restrictions and with ethical aspects.

MATERIALS AND METHODS

AG Procurement and Purification

We purchased raw pharmaceutical grade AG powder (Stern Italia, Milano, Italy) that was then dissolved in sterile pyrogen-free, deionized water (Italian Pharmacopeia) over 24 to 36 hours in the dark at room temperature; 3% NaCl (Italian Pharmacopeia) was then added to adjust the osmolality to physiological levels and the

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pH was also adjusted to 7.4. The solution underwent multiple sequential passages through methylcellulose and polyester filters (CUNO Italy, Benevento, Italy), dialysis and solution reconstitution, followed by final filtration (0.2 μ m) to ensure sterility. The final 1.6% solution was stored in the dark room at 4°C to avoid AG depolymerization. Endotoxin levels in this preparation were measured by the limulus amoebocyte lysate method (Cambrex, Brussels, Belgium).

Human Islet Cell Harvesting

Human islets procured by the Central Southern Italian Transplant Organization were separated and purified from cadaveric donor pancreata, according to the Edmonton protocol.⁴ Only a few preparations would be considered for transplant: the final islet yield must be equal to or greater than 400,000 islet equivalents with a purity and viability in excess of 80%. Such a preparation would be culture-maintained for 24 hours in HAM F12 (Celbio, Milano, Italy), supplemented with 10% human albumin (Celbio) at 37°C. Thereafter the islets were carefully washed to remove proteins in preparation for microencapsulation. At 24-hour isolation, batches of 20 human islets underwent static incubation with glucose at various concentrations to assess glucose-stimulated insulin release (GSIR), a marker of islet function.

Microencapsulation of Human Islets

Briefly, the islet tissue pellet, usually amounting to a few tens of a milliliter, was thoroughly mixed with the 1.6% AG solution, so as to make a final homogeneous suspension. The AG/islet proportion was adjusted so that one capsule would contain one islet, with fewer than 5% empty capsules. The suspension was extruded through a microdroplet generator, combining air shears with mechanical pressure; the AG droplets were collected in 1.2% CaCl₂ (Sigma Aldrich, Milano, Italy) immediately turning into gel microbeads. They were sequentially overcoated with PLO and an outer AG layer. The final microcapsule preparations, which did not exceed a final volume of 50 mL, were incubated for additional 24 hours for sterility and viability checking with ethidium bromide + fluorescein diacetate (Sigma) using fluorescence microscopy.

Peritoneal Injection Procedure

A minimally invasive microcapsule injection method was devised in collaboration with our Radiology and Imaging Department. Under abdominal echography with local anesthesia, the peritoneal cavity was imaged and normal saline (Italian Pharmacopeia) injected to map and detect the capsule deposit area within the peritoneal leaflets. The capsule suspension was diluted in 100 mL of saline (total final volume = 150 mL) and painlessly delivered with 60-mL plastic syringes, 16-G needle (BD, Milano, Italy) over 10 to 15 minutes. The entire procedure was meticulously reported to the Italian Ministry of Health, during several audits, to discuss and make appropriate changes. The clinical study was conducted at the University of Perugia Hospital, Departments of Internal Medicine and Radiology, with the patient being monitored after transplant for 1 week.

RESULTS

After 3 years of accurate evaluations, in compliance with laws regulating good medical practice on phase I clinical trials and deliberations of the Perugia University Hospital



Fig 1. Photomicrograph of microencapsulated human islets (light microscopy, 10 $\times$).

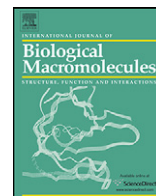
Board and ethical committee, including appropriate informed consent text, the Ministry of Health has released us to initiate a closed, pilot phase I clinical trial of microencapsulated human islet allografts into 10 nonimmunosuppressed patients with T1DM.

Most of the procured human pancreata did not yield the required islet mass due to unfavorable organ preservation and storage conditions, long ischemia time, or poor tissue digestion, the latter likely related to collagenase batch variability. The islet preparations fulfilling quality control criteria, in terms of morphology, viability, and function (GSIR), were encapsulated in AG/PLO. Each capsule (Fig 1) usually contained one human islet with few empty microcapsules. If and when exceeding 5% of the total, the empties were removed by handpicking. No changes of morphological, functional, and sterility parameters were observed over the in vitro culture maintenance period (total 72 hours) pretransplantation. The microcapsule infusion procedure was easy and painless for the patients who could stand up a few minutes, upon the procedure's completion. No side effects during or after the procedure were reported in the two first cases.

In conclusion, even if eight patients still need to be done, grafting of microencapsulated islets seems to be safe and devoid of side effects. Hence this could be a viable method to implant islets with no recipient pharmacological immunosuppression. Microencapsulation also fosters hope that nonhuman tissue may be employed, in the future, for grafting of patients with T1DM.

REFERENCES

1. Calafiore R: Alginate microcapsules for pancreatic islet cell immunoprotection: struggle and progress towards the final cure for type 1 diabetes mellitus. *Expert Opin Biol Ther* 3:201, 2004
2. Calafiore R, Basta G, Luca G, et al: Grafts of microencapsulated pancreatic islet cells for the therapy of diabetes mellitus in non-immunosuppressed animals. *Biotechnol Appl Biochem* 39:159, 2004
3. Duvivier-Kali VF, Omer A, Parent RJ, et al: Complete protection of islets against allojection and autoimmunity by a simple barium membrane. *Diabetes* 50:1968, 2001
4. Shapiro AMJ, Lakey JRT, Ryan EA, et al: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230, 2000



Influence of the extraction–purification conditions on final properties of alginates obtained from brown algae (*Macrocystis pyrifera*)

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ABSTRACT

In this work, three methods (ethanol, HCl, and CaCl₂ routes) of sodium alginate extraction–purification from brown seaweeds (*Macrocystis pyrifera*) were used in order to study the influence of process conditions on final properties of the polymer. In the CaCl₂ route, was found that the precipitation step in presence of calcium ions followed by proton-exchange in acid medium clearly gives alginates with the lowest molecular weight and poor mechanical properties. It is well known that the acid treatment degrade the ether bonds on the polymeric chain. Ethanol route displayed the best performance, where the highest yield and rheological properties were attained with the lowest number of steps. Although the polymer I.1 showed a molar mass and polydispersity index (M_w/M_n) similar to those of commercial sample, its mechanical properties were lower. This performance is related to the higher content of guluronic acid in the commercial alginate, which promotes a more successful calcium chelation. Moreover, the employment of pH 4 in the acid pre-treatment improved the yield of the ethanol route, avoiding the ether linkage hydrolysis. Therefore, samples I.2 and I.3 displayed a higher M_w and a narrower distribution of molecular weights than commercial sample, which gave a higher viscosity and better viscoelastic properties.

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1. Introduction

Although the early state of biosynthesis of carbohydrates in seaweeds is similar than in plants, some of the polysaccharides finally synthesized are found only in seaweeds. These biopolymers are present principally in the cell wall, giving mechanical resistance to algae. Properties such as flexibility and softness in water to support currents are more important than rigidity. Therefore, the polysaccharides that prevail in seaweed are those that possess properties of gels and mucilage, being cellulose also present in a lower amount [1–6].

There are three principal groups of seaweeds classified by their colour: brown, red and green seaweeds. Each of them possesses the predominance of a typical polysaccharide [7], being alginate the most abundant in marine brown seaweed. Although there are different species of brown seaweed that contain alginates, these are not sufficiently abundant and suitably located for commercial production. The species of brown seaweed most commercially exploited are: *Laminaria hyperborea*, *Macrocystis pyrifera* and *Ascophyllum nodosum*. These species are mainly manufactured in countries such as USA, Japan, China, France, and Norway [8].

In natural environments, alginates exist in the cell wall as a mixture of calcium, potassium and sodium salts of alginic acid [9]. They constitute a family of linear copolymers of (1 → 4) β-D-mannuronic acid (M) and (1 → 4) α-L-guluronic acid (G) units. The chemical composition and sequence of M and G units depend on the biological source, growth, and stationary conditions. These polysaccharides have three types of diad sequences, i.e. MM, GG and MG blocks [10–12]. Sodium alginate is a water-soluble polymer, which gives highly viscous solutions. It can be used as a stabilizer of suspensions and as thickener in food industries among other applications [9]. Another characteristic property of the sodium alginate solutions is the ability of gel formation in the presence of polyvalent cations, such as Ca²⁺ [13]. Generally, the extraction and purification processes of alginates are based on the conversion from the insoluble form in the plant cell walls to the soluble one, normally the sodium salt, followed by successive dissolutions and precipitations to eliminate impurities [14,15]. Alginates extraction from brown seaweed has been studied during several decades in order to develop economic systems, obtaining high yields and a controlled molecular weight for different applications [16–18]. In some South American countries such as Argentina, there is no industry producing alginates despite the large amount of brown seaweed such as *M. pyrifera* along of its seacoast [19]. In this work we present a comparative analysis of three routes of sodium alginate purification in order to have a product with controlled molar mass and degree of

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purity. Samples of brown seaweeds were collected from Patagonian Argentine coast and used as raw material to carry out this study. Extracted alginate was characterized by SEC, ^1H NMR, rheological studies, ability to gel formation and swelling degree. On the other hand, the characteristic properties of extracted sodium alginates were studied and compared with those of a commercial sample.

2. Experimental

2.1. Materials and reagents

The raw material from which the alginate was extracted consisted of sheets and stems of brown seaweed (*M. pyrifera*) from Patagonian Argentine coast, which were dried, previously crushed and sifted (10–20 mesh). The following chemicals were also purchased and used in the extraction–purification processes: HCl 37 wt% (Cicarelli, Argentine); NaOH p.a. (Anedra, Argentine);

Na_2CO_3 p.a. (Anedra, Argentine); CaCl_2 p.a. (Anedra, Argentine); ethanol 96% (Carries, Argentine); diatomaceous earth (Anedra, Argentine); eriochrome black-T p.a. (Fluka, Switzerland). A commercial sample of sodium alginate (N° 71238 Fluka, Switzerland) was employed as a reference. In addition, a solution of eriochrome black-T (5 g per 1 L of ethanol) was prepared, adding then 5 mL of the last one to 5 mL of a 0.5 M $\text{NH}_4\text{Cl}/\text{NH}_3$ buffer solution at pH 10, in order to obtain the indicator solution used for qualitative detection of calcium ions.

2.2. Extraction of alginates from seaweed

A crushed sample (10 g) of dry seaweed was moistened by addition of distilled water (400 mL) and a 0.1 N HCl aqueous solution was added, under high stirring, in order to have a pH 4, following a similar extraction method reported by Arvizu Higuera et al. [16]. This mixture was stirred during 15 min at room temperature

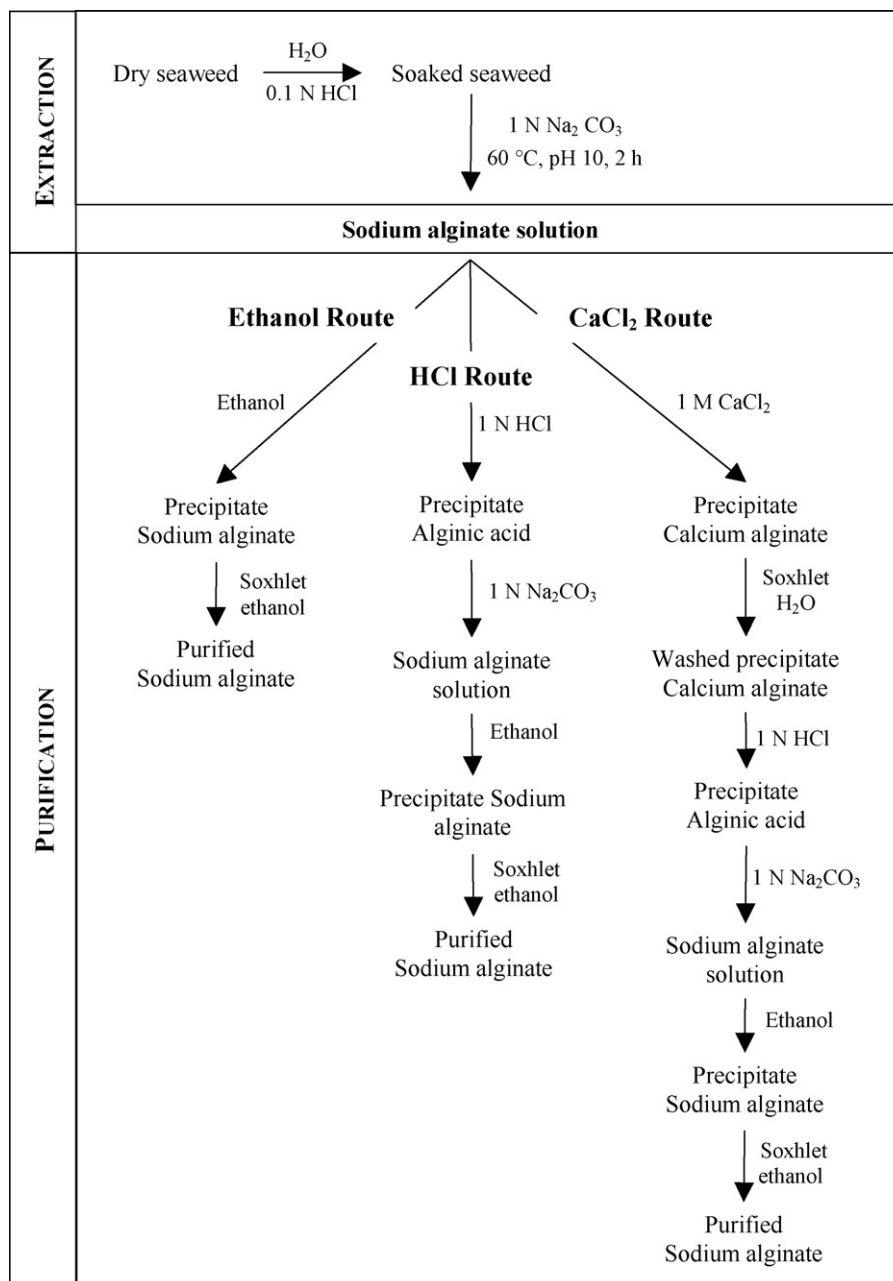


Fig. 1. Scheme corresponding to the three routes of alginate purification.

and then the supernatant was eliminated. This pre-treatment was repeated three times with every sample, using each time 40, 25 and 20 mL of HCl solution, respectively. Then, the moistened seaweed was placed in a beaker with 250 mL of a 1N Na_2CO_3 solution (pH 11.5) and mechanically stirred at 60 °C for 2 h. The extracted sodium alginate from the initial material was diluted to 800 mL with distilled water and 1 g of diatomaceous earth was added, stirring this mixture during 15 min. The insoluble material was separated by centrifugation, obtaining then sodium alginate in the supernatant.

2.3. Purification of sodium alginate extracts

Three different processes of purification were used with the solution of sodium alginate obtained in the above process, which have been named route of ethanol, HCl, and CaCl_2 , respectively. A schematic representation of the steps developed in the processes is shown in Fig. 1.

2.3.1. Ethanol route (I)

Aqueous solution of sodium alginate obtained in the extraction step was directly precipitated, under stirring, by addition of ethanol until reaching a proportion 1:1 in volume, respectively. Thus, the insoluble polymer was separated and then exhaustively washed with ethanol by soxhlet for 100 h. Finally, the biopolymer was dried at room temperature under vacuum until constant mass.

2.3.2. HCl route (II)

In this route of purification, the solution of extracted sodium alginate and 250 mL of 1N HCl were mixed at room temperature under stirring for 1 h (pH 2). Next, the precipitate of alginic acid was separated by centrifugation adding later to it 100 mL of distilled water and 150 mL of 1N Na_2CO_3 . Then, this mixture was left under stirring at room temperature for 1 h in order to obtain the soluble form sodium alginate. Later, the polymer was precipitated from this solution by slow addition of ethanol (1/1 volume ratio), employing the same procedure of washing and drying described in Section 2.3.1.

2.3.3. CaCl_2 route (III)

The aqueous solution of sodium alginate was precipitated by the addition of 1 M CaCl_2 (200 mL). The precipitate of calcium alginate was exhaustively washed with distilled water using a soxhlet for 64 h. Then, 150 mL of distilled water was added to the precipitate followed by the addition of 1N HCl until pH 2 in the supernatant, maintaining this mixture under agitation at room temperature for 3.5 h. The insoluble material obtained (alginic acid) was separated from the supernatant by centrifugation. Next, the alginic acid was washed with 1.5 L of 0.05N HCl under stirring at room temperature, without detecting the presence of Ca^{2+} in the supernatant when it was analysed by eriochrome black-T indicator. Later, the precipitate of alginic acid was treated as in Section 2.3.2.

2.4. Calcium determination assays

Qualitative analysis of the presence of calcium ion in the supernatant was performed in the CaCl_2 route. Here, an indicator solution of eriochrome black-T (1 mL) was mixed with an equal volume of the sample supernatant, which changes its colour from blue to pink in the presence of calcium ions. Quantitative determination of the sodium and calcium content on the alginates samples were performed at the CNRS central microanalysis service at Solaize (France).

2.5. Molecular weight distribution

Purified alginates isolated under the sodium salt form were characterized by size exclusion chromatography (SEC) using a Waters Alliance GPCV2000 (USA) equipped with three detectors on line: a differential refractometer, a viscometric detector, and a multi angle laser light scattering (MALLS) detector from Wyatt (USA). The concentration of polymer injected was in the range of 0.5–5 g/L (depending on the range of molecular weights), with an injection volume of 108 μL using two columns in series (Shodex OH-pack 805 and 806). The dn/dc adopted for the analysis was 0.165. All the samples were filtrated on a 0.2 μm pore membrane ("Sartorius AG" cellulose acetate filter) before injection, in order to retain large aggregates. The eluent used was a 0.1 M NaNO_3 aqueous solution, at 30 °C as elution temperature and a flow rate of 0.5 mL/min; the molecular weight distribution, weight-average molecular weight (M_w), polydispersity index (M_w/M_n), and intrinsic viscosity of the eluted polymers were obtained as characteristics of the biopolymers.

2.6. Chemical structure

Chemical composition of extracted alginates was obtained by NMR. Polymer samples were dissolved in D_2O with a concentration 6 mg/mL. ^1H NMR experiments were performed using a Bruker AC-300 (Germany) spectrometer. The delay adopted was 20 s. Chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm). Deuterium oxide was obtained from SDS (Vitry, France). ^1H NMR spectroscopy is suitable for characterizing both the composition and the distribution sequence of the two-uronate residues in alginate samples [21]. We have assigned the signals at 5, 4.6, and 4.37 ppm, like those corresponding to $\text{H}_1\text{-G}$ (pick 1), $\text{H}_1\text{-M} + \text{H}_5\text{-GM}$ (pick 2), and $\text{H}_5\text{-GG}$ (pick 3), respectively (Fig. 2). Taking into account that protons areas of $\text{H}_1\text{-G}$ and $\text{H}_5\text{-G}$ are equals, $\text{H}_5\text{-GM}$ is attained from the difference between the picks 1 and 3. Then, making the difference between the pick 2 and $\text{H}_5\text{-GM}$, the $\text{H}_1\text{-M}$ area is obtained. From these values, the M/G ratio is calculated as $\text{H}_1\text{-M}/\text{H}_1\text{-G}$ and the fraction GG as $\text{H}_5\text{-GG}/\text{H}_1\text{-G}$.

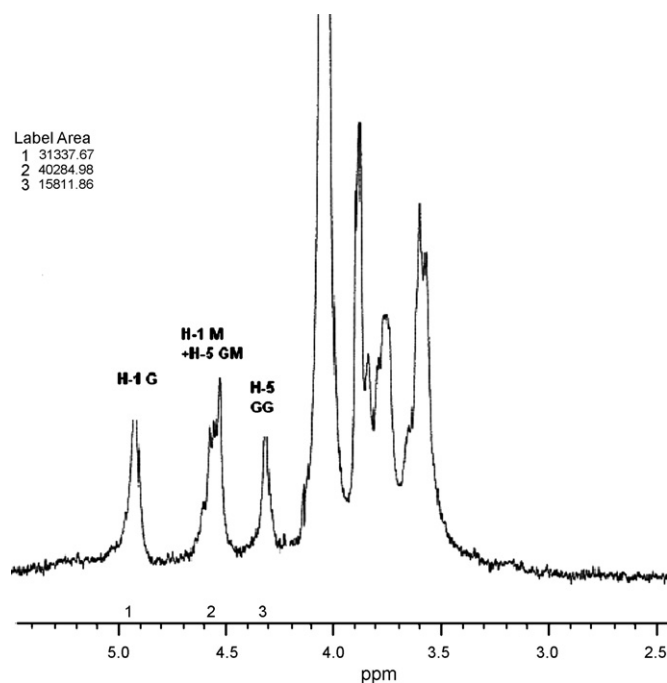


Fig. 2. ^1H NMR spectrum of sample I.1 in deuterium water and its characteristic signals.

2.7. Gelation ability of the polymeric backbone in the presence of calcium ions and the degree of swelling

Gelation ability of sodium alginate solutions in the presence of calcium ions was carried out by dialysis through a porous cellulosic membrane “Spectra/Por” (exclusion limit $M = 12\text{--}14,000$) [20]. Here, a sodium alginate aqueous solution at a concentration of 20 g/L was prepared and then introduced (20 mL) in the dialysis bag against a 1 M CaCl_2 solution during 48 h to assure equilibrium. A slice of gel (near 4 mm thick) was cut and placed between the plates of the rheometer and its rheological properties were measured. Moreover, the swelling degree (SD) of the gel was obtained and expressed as grams of regained water per gram of dry sodium alginate. Swelling in water was determined by difference of weight between the swollen gel and the dry mass of sodium alginate (based on the amount of polymer introduced in the bag). Later, the gel formed in excess of CaCl_2 was dried, and then swollen again in water until reaching equilibrium. The swelling liquid was removed, repeating the practice 5 times in order to eliminate the excess of CaCl_2 included, and then the re-swelling degree (RSD) was measured. Here, the gel was weighted and dried at 70 °C until achieve constant mass, and then RSD was expressed as grams of water per gram of dried polymer under calcium form.

2.8. Rheology of the sodium alginate solutions and the calcium alginate gels

The rheological behaviour of the solutions and gels was determined using an AR 1000 rheometer from “TA Instruments” (USA) at 25 °C. Parallel plate geometry with 2 cm diameter plate was used for gels, and a cone and plate geometry (4 cm diameter plate with 3.59° cone) for solutions. Dynamic experiments were performed in the linear viscoelastic region. Dynamic moduli (G' and G'') as well as complex viscosity $|\eta^*|$ were determined as a function of the angular frequency (ω).

3. Results and discussion

Brown seaweeds (*M. pyrifera*) from Patagonian Argentine coast were used in a study of extraction and purification of sodium alginate in order to attain the highest molar mass and purity degree. The products were obtained by three ways, named “Ethanol (I)”, “HCl (II)”, and “ CaCl_2 (III)” route. Acid pre-treatment was used in order to eliminate polyvalent cations. After that, the soluble polymer diffuses out of the seaweed under basic conditions, and then it is purified by different ways. The final products were analysed and their chemical composition as well as physical properties were compared.

3.1. Purification process of sodium alginate

Purification process is based on successive solubilizations and precipitations of the polymer in aqueous medium. Different conditions are employed to eliminate the soluble impurities and all multivalent counter ions in order to obtain the polymer under sodium salt form. As can be seen in Fig. 1, there is a step corresponding to alginate extraction using a Na_2CO_3 solution at 60 °C, in which cellular matrix is hydrolysed and the alginate polymer is liberated to the solution followed by different purification ways. For most of the cases, the alginate yields were higher than other values published previously [14,19]. Fig. 1 and Table 1 show a logic effect of the number of steps and the extraction conditions on purity and extracted polymer yield. Moreover, the highest yield of purified sodium alginate was obtained by ethanol route, which presents the lower number of purification steps (Table 1). This behaviour reflects the already well-known low stability of the links of these

Table 1

Performance of the extraction–purification routes of sodium alginate.

Sample	Purification		Ca^{2+} content (ppm)	Extraction yield (wt%)
	Route	Number of steps		
I.1	Ethanol	2	2300	33
I.2			–	34
I.3			–	32
II.1	HCl	4	–	25
II.2			2300	29
III.1	CaCl_2	6	1500	27
III.2			–	29

Calcium content for commercial sodium alginate was 1200 ppm.

polysaccharides principally in acid medium and in less extent in basic conditions. In addition, the extraction yield values given in Table 1 were calculated on the basis of corrected dry weight of the samples without the extra materials such as sand occluded in the initial algae.

3.2. M/G composition of the polymers and calcium content

The chemical composition of the extracted sodium alginate was determined using ^1H NMR spectroscopy. As can be seen in Fig. 2, the signal at 4.95 ppm corresponds to the H-1 of guluronic units, whereas the H-5 of guluronic units in GG block appears at 4.31 ppm, and the large signal between 4.45 and 4.7 ppm belongs to H-1 of mannuronic units and H-5 of guluronic units in GM moieties as assigned previously [21]. From the integration of these signals both the M/G ratio and the fraction of G units included in GG blocks were estimated. The results are given in Table 2, showing that M/G ratios of alginates obtained from *M. pyrifera* are higher than the value corresponding to the commercial sample, since the last one was probably extracted from a different algae species. Table 2 also shows that an average value of $\text{M/G} \sim 1.15$ is found for the polymers purified through different ways, from which a large fraction of G units (0.7) is involved in GG blocks. No significant dispersion in this ratio was obtained for the three routes. On the other hand, Table 1 indicates that the residual calcium content in the samples (1500–2000 ppm) was close to the value obtained for the commercial alginate (1200 ppm). Relatively lower values of calcium content were obtained when acid route was employed. An increase in the proton concentration lead to a higher polycations exchange, and then a product with a higher purity can be obtained. However, this variation gives also a decrease of the average molecular weight by hydrolysis of the ether linkages.

3.3. Molecular weight distribution

Molecular weight distribution of the samples was determined by SEC at 30 °C. Weight-average molecular weight (M_w) and the polydispersity index (M_w/M_n) are given in Table 2, where it is possible to observe that the best purification condition is the direct precipitation by ethanol. Ethanol route gives the highest weight-average molecular weight (M_w) with a relatively low polydispersity. Moreover, the molecular weight distribution of the extracted alginates was compared with the commercial sample (Fig. 3). Here, the maximum in the molecular weight distribution moves to higher molar mass when a lower number of steps in the purification route are used, which can be related to chains degradation. In addition, from SEC measurements it was possible to estimate the percentage of soluble components in each sample. Table 2 shows that the soluble percentage of every extracted alginate by ethanol and acid routes was higher than 60%, which is related to a successful purification of the polymer. Intrinsic viscosity data shown in Fig. 4 and Table 2, indicate that Mark Houwink exponent is, in the range of molecular

Table 2
Rheological behaviour and characterization by SEC and ^1H NMR of the different alginates.

Sample	GG fraction	M/G	$M_w \times 10^3$ [g/mol]	M_w/M_n	Soluble % (SEC)	$[\eta]$ (mL/g) (SEC)	$ \eta^* ^a$ (0.1 Hz) [Pa s]
Commercial	0.48	0.43	279	1.9	70	660	1.07
I.1	0.69	1.17	297	1.8	72	575	0.60
I.2	–	–	350	1.6	61	1100	–
I.3	–	–	396	1.5	62	1185	–
II.1	0.76	1.29	227	1.6	69	530	0.39
II.2	0.59	1.30	213	1.6	70	480	0.19
III.1	0.69	0.97	94	3.0	57	170	0.02
III.2	0.77	1.12	56	1.9	58	150	0.01

^aSample in water solution (20 g/L; 25 °C), being (I), (II) and (III) the purification routes of ethanol, HCl and CaCl_2 , respectively.

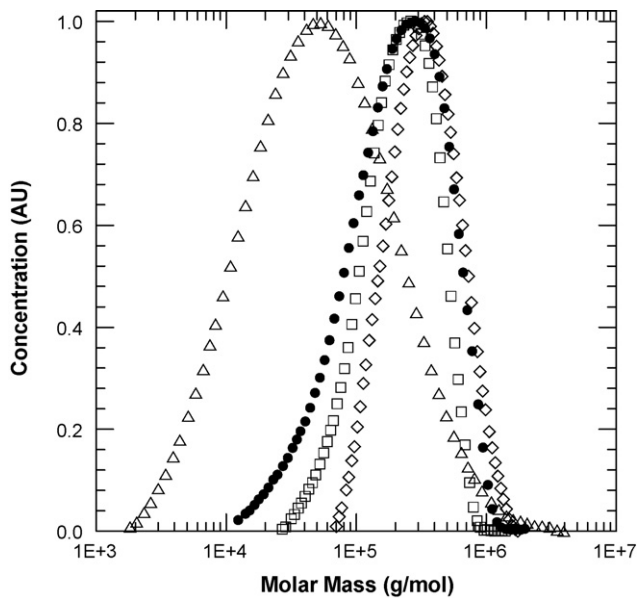


Fig. 3. Molecular weight distributions obtained by size exclusion chromatography. Symbols: (●) commercial; (◇) I.3; (□) II.1; (△) III.1.

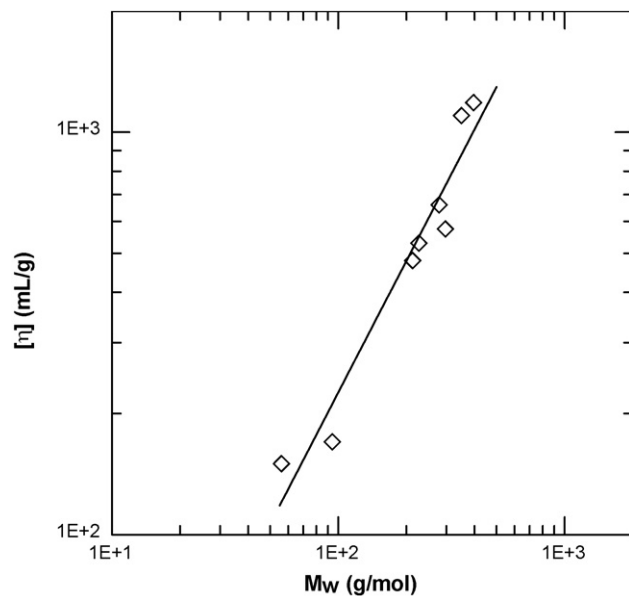


Fig. 4. Intrinsic viscosity $[\eta]$ in 0.1 M NaNO_3 as a function of weight-average molar mass (M_w) for extracted sodium alginates; all the experimental data are obtained from SEC (Table 2).

weight analysed, $\alpha = 1.09$. This value is in good agreement with that determined in a previous work ($\alpha = 0.97$ in 0.1 M NaCl) [22]. Then, it is possible to confirm that alginates are semi-rigid molecules, which can be characterized by a persistence length depending slightly on their composition as discussed previously [23].

3.4. Rheology

Rheology of polymer solutions at 20 g/L in water was tested in steady state and dynamic experiments. All the solutions have a Newtonian plateau at low shear rate. As it was expected, loss modulus (G'') is always higher than storage modulus (G') in the range of frequency covered [24]. An example is given in Fig. 5, where the behaviour of the commercial and I.1 sample is compared, showing the former a higher performance as thickener. From these rheological data, it can also be concluded that the direct precipitation in the purification of sodium alginate through “ethanol route” gave the extracted polymer solution with the highest viscosity (Table 2). Given that the polymeric chain degradation is high in presence of HCl solution during the acid pre-treatment of the algae before to the extraction process, the acidity must also be carefully kept at pH 4. Moreover, the purification by the “HCl route” gives, in both samples (II.1 and II.2), relatively similar results with only few aggregates as it can be seen from soluble % (Table 2). Nevertheless, the use in this route of an acidic treatment produces hydrolysis of some linkages with a decrease in the molecular weight and a lower rheological performance for these samples.

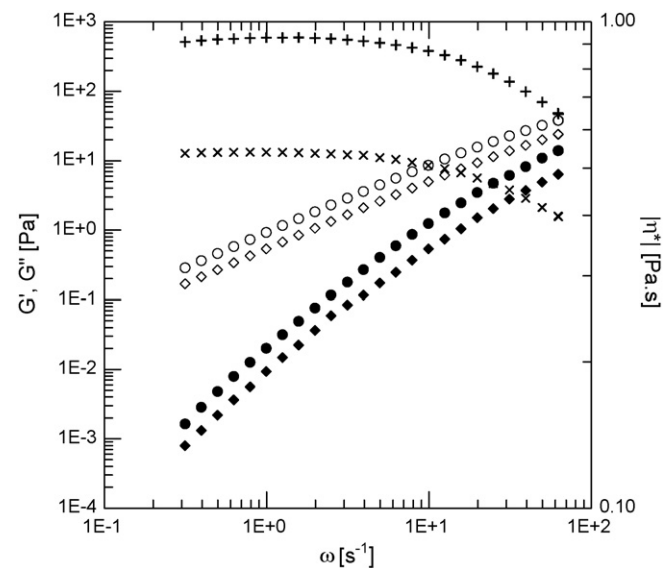


Fig. 5. Dynamic rheological behaviour of 20 g/L sodium alginate aqueous solutions performed at 25 °C. Elastic and viscous moduli, and complex viscosity: (●, ○, ×) commercial; (◆, ◇, ×) I.1 sample.

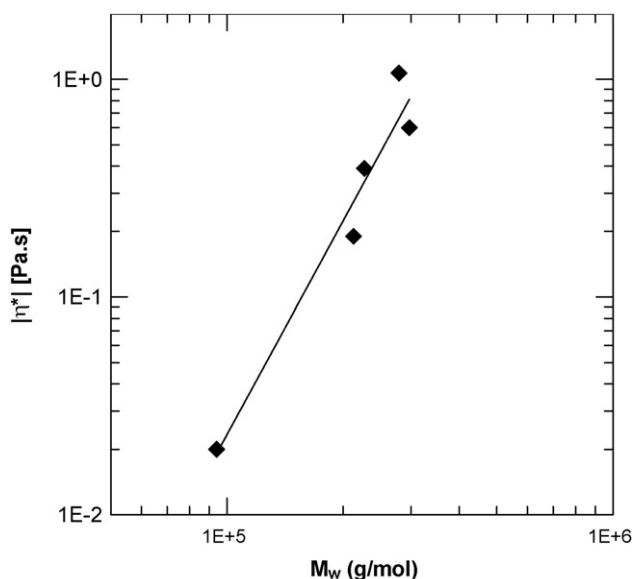


Fig. 6. Complex viscosity measured at 0.1 Hz for alginate solutions (20 g/L in water) as a function of weight-average molar mass (M_w) for extracted sodium alginate. Slope 3.26.

On the other hand, the “CaCl₂ route” has the highest number of purification steps, including an acid treatment (Fig. 1), giving the worst results (III.1 and III.2 samples). Table 2 shows that samples III present the lowest molecular weight and viscosity with a lot of aggregates and a relatively high content of the residual calcium ions. The complex viscosity as a function of the angular frequency at 0.1 Hz for the different samples was obtained, and then their values compared (Table 2). There are not enough data to determine the critical value of the average molecular weight for effect of entanglement coupling on viscosity (M_c). Nevertheless, the values of weight-average molecular weight obtained seem to be over M_c , at the polymer concentration used (20 g/L in water), which screens the long range electrostatic repulsions. Viscosity values as a function of weight average molecular weight show a power trend (Fig. 6) with a slope of approximately 3.26 as it is expected at high molecular weights, due to the entanglement coupling [25].

3.5. Gelation ability of the polymers

Gelation ability of sodium alginate polymers was evaluated using a 20 g/L solution of polymer against a 1 M CaCl₂ solution for 48 h. Rheological properties of formed gels were obtained by dynamic measurements in parallel plate geometry. Fig. 7 shows the evolution of dynamic moduli (G' and G'') as a function of the frequency for formed gels of commercial, I.1 and I.3 sample. An elastic modulus (G') higher than the viscous one (G'') in all the range of frequency measured is obtained. From the relationship between the polymeric chain and gel properties (Tables 2 and 3), it is possible to observe that viscoelastic properties are dependent on both the chemical composition and the polymer molar mass. Those alginates with a lower M/G ratio displayed a higher G' value (Table 3) as result of a higher degree of calcium chelation, which is directly related to a higher content of guluronic groups. Although the moduli are lower when GG fraction is higher (Tables 2 and 3, Fig. 7), the total GG fraction (TGF) of the commercial sample (36%) is higher than sample I.1 (32%). This is in agreement with the “egg box” binding model of calcium by polyguluronate. The TGF % value was calculated from M/G ratio and GG-block fraction (Table 2), where $TGF\% = [100 \times GG\text{-block fraction}] / [(M/G) + 1]$ was expressed as the percentage of G units presents in the GG block fraction referred to the total monomer (M + G) content.

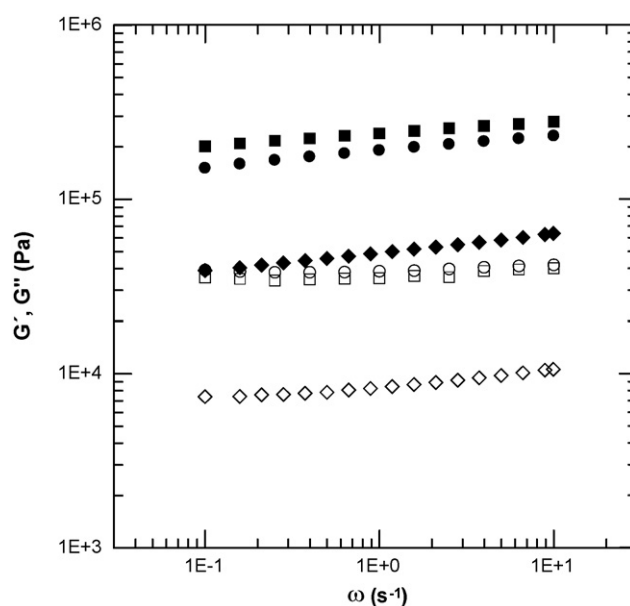


Fig. 7. Dynamic rheological behaviour of calcium alginate gels at 25 °C. Elastic and viscous moduli of (●, ○) commercial, (◆, ◇) I.1 and (■, □) I.3 samples.

Table 3

Viscoelastic properties at 25 °C and swelling degree of calcium gels obtained from aqueous solution of alginates with a Cp = 20 g/L.

Sample	G' (at 1 Hz) [Pa]	G'' (at 1 Hz) [Pa]	SD ^a SD ^b	
			[g H ₂ O/g dry polymer]	
Commercial	191,200	38,000	25	4.8
I.1	51,800	8,800	24	4.7
I.2	295,000	55,000	26	5.3
I.3	240,000	38,200	24	5.4
II.1	43,400	7,100	26	4.4
II.2	49,400	8,300	27	5.2
III.1	50,600	7,900	30	5.3
III.2	38,900	5,400	31	6.0

^a Swelling degree.

^b Re-swelling degree.

On the other hand, the control of acidity at pH 4 during acid pre-treatment allowed attaining the samples I.2 and I.3 with a very good reproducibility. These alginates (Table 2 and Fig. 3) displayed a narrower molecular weight distribution and a higher molar mass than the commercial sample. Therefore, the gelation ability of the extracted polymers was improved, showing the highest viscosity and improved viscoelastic properties (Tables 2 and 3). Extracted alginates with a higher molecular weight form stronger gels (Fig. 7) due to an increase in the number of cross-linking points (calcium chelation). On the other hand, the lower values of swelling and re-swelling degrees obtained with samples I.1 to I.3 also indicates a higher degree of cross-linking in those gels.

4. Conclusions

In this work a basic extraction of sodium alginate from brown seaweeds (*M. pyrifera*) was performed in order to examine the influence of three purification methods on the yield and final properties of the polymer. Clearly the use of a step of precipitation in presence of calcium ions followed by the cations exchange in acid medium gives alginates with poor viscoelastic properties. Acid treatments degrade the polymer chain when it is used in the process. Ethanol route results the most successful process using the lowest number of steps. Direct polymer precipitation with ethanol (1/1 volume ratio) leads to the best yield. Sample I.1 with an weight

average molecular weight and M_w/M_n similar to the commercial sample shows lower viscoelastic properties, both in solution and in gel form. This behaviour can be explained considering the higher G unit content (TGF %) in the commercial alginate, which promotes a more successful calcium chelation. A pH higher than 3.5 in the acid pre-treatment enhanced the “ethanol route”, avoiding the rupture of ether linkages. Then, extracted alginates I.2 and I.3 were obtained with a higher M_w and a narrower molecular weight distribution, presenting rheological properties comparable to or even better than the commercial sample.

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References

- [1] N.P. Chandía, B. Matsuhira, A.E. Vásquez, *Carbohydrate Polymers* 46 (2004) 81.
- [2] A. Sharma, M.N. Gupta, *Carbohydrate Polymers* 48 (2002) 391.
- [3] T. Gotoh, K. Matsushima, K. Kikuchi, *Chemosphere* 55 (2004) 135.
- [4] O. Holte, E. Onsoyen, R. Myrvold, J. Karsen, *European Journal of Pharmaceutical Sciences* 20 (2003) 403.
- [5] M. Simeone, A. Alfani, S. Guido, *Food Hydrocolloids* 18 (2004) 463.
- [6] J. Rhim, *Lebensm.-Wiss. U.Technol.* 37 (2004) 323.
- [7] E. Percival, R.H. McDowell, in: P.M. Dey (Ed.), *Methods in Plant Biochemistry*, vol. 2, Academic Press, New York, 1990.
- [8] M. Rinaudo, *L'actualité Chimique* (November–December) (2002) 35.
- [9] J. Sime Wilma, in: P. Harris (Ed.), *Alginates, Food Gels*, Elsevier Science Publishers Ltd., England, 1990.
- [10] A. Haug, B. Larsen, *Acta Chemica Scandinavica* 16 (1962) 1908.
- [11] E.D.T. Atkins, W. Mackie, K.D. Parker, E.E. Smolko, *Polymer Letter* 9 (1971) 311.
- [12] FAO, Sodium alginate, calcium alginate, alginic acid, 49th JECFA 1997, www.fao.org.
- [13] K.I. Draget, G. Skjåk-Bræk, O. Smidsrod, *International Journal of Biological Macromolecules* 21 (1997) 47.
- [14] B. Larsen, D.M.S.A. Salem, M.A.E. Sallam, M.M. Mishrikey, A.I. Beltagy, *Carbohydrate Research* 338 (2003) 2325.
- [15] D.J. McHugh, *FAO Fisheries Technical Paper* (2003) 441.
- [16] D.L. Arvizu Higuera, G. Hernández Carmona, E. Rodríguez Montesinos, *Ciencias Marinas* 23 (2) (1997) 195.
- [17] G. Hernández Carmona, M.M. Casas Valdez, *Investigaciones Marinas* 13 (1985) 18.
- [18] G. Hernández Carmona, M.M. Casas Valdez, M. Aguirre Vilchis, *Investigaciones Marinas* 19 (1991) 259.
- [19] M. Ferrario, E. Sar (Eds.), *Macroalgas de Interés Económico. Cultivo, Manejo, Industrialización*, Editorial de la UNLP, La Plata, Argentina, 1996.
- [20] G. Skjåk-Bræk, H. Grasdalen, O. Smidsrød, *Carbohydrate Polymers* 10 (1989) 31.
- [21] A. Heyraud, C. Gey, C. Léonard, C. Rochas, S. Girond, B. Kloarec, *Carbohydrate Research* 289 (1996) 11.
- [22] M. Rinaudo, D. Graebing, *Polymer Bulletin* 15 (1986) 253.
- [23] (a) M. Rinaudo, *Polymer Bulletin* 27 (1992) 585;
(b) M. Rinaudo, in: J. Kahovec (Ed.), *Macromolecules*, vol. 207, VSP, 1992.
- [24] M.A. Rao, *Rheology of Fluid and Semisolid Foods. Principles and Applications*, Aspen Publishers, Inc., Maryland, USA, 1999.
- [25] J.D. Ferry, *Viscoelastic Properties of Polymers*, John Wiley & Sons, New York, USA, 1970.