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Departamento de Ingeniería Química y

Tecnología del Medio Ambiente

Obtención de ácidos orgánicos por fermentación de subproductos lácteos

TESIS DOCTORAL

POR

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// AGRADECIMIENTOS

El largo y tortuoso camino parece que llega su fin pero alcanzar tal meta habría sido imposible sin la inestimable e infinita ayuda de varias personas.

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RESUMEN

El creciente interés comercial experimentado en los últimos años por los ácidos orgánicos ha estimulado la producción biotecnológica de los mismos como una solución integral a la paulatina demanda suscitada en este segmento químico. De igual modo, la sostenibilidad ha sido otro de los retos afrontados por la biotecnología industrial, la cual se ha centrado tanto en el desarrollo de estrategias eficientes, como en el aprovechamiento biotecnológico de residuos o subproductos industriales. En este contexto, el presente trabajo ha abordado la producción biotecnológica de dos ácidos orgánicos con importante relevancia comercial como son el ácido láctico y lactobiónico a partir de dos residuos alimentarios, el suero lácteo de yogurt y de queso.

En primer lugar se ha estudiado la producción de ácido láctico por *Lactobacillus casei* a partir de los azúcares fermentables presentes en el suero lácteo de yogures retirados de la cadena de comercialización. Así, se ha evaluado la capacidad fermentativa en términos de rendimiento, evolución y eficiencia de un sistema fermentativo caracterizado por la presencia de glucosa, sacarosa y lactosa como substratos. Con el objetivo de profundizar en el comportamiento fermentativo, se ha empleado asimismo la citometría de flujo multiparamétrica como herramienta para la evaluación de la funcionalidad celular durante el bioproceso. De hecho, la aplicación de esta técnica de cultivo independiente ha permitido conocer el status fisiológico y la robustez celular del sistema a nivel poblacional.

Asimismo, se ha estudiado la bio-producción de ácido lactobiónico por *Pseudomonas taetrolens* a partir de suero lácteo bajo diferentes condiciones operacionales. El ácido lactobiónico ha irrumpido recientemente en el mercado con una pléyade de aplicaciones en ámbitos tan diversos como el alimentario y el biomédico debido a sus excelentes propiedades. Hasta la fecha su producción industrial está restringida a la catálisis vía química, resultando en una metodología ineficiente e insostenible debido al uso de costosos catalizadores y a la formación indeseable de subproductos laterales durante la reacción. La producción biotecnológica puede resolver estas limitaciones así como posicionarse como la alternativa productiva más adecuada ante el incremento en la demanda del ácido lactobiónico. El bioproceso desarrollado en el presente trabajo ha sido llevado a cabo en diferentes escalas con el fin de localizar y poner en evidencia las etapas limitantes, optimizando el sistema en base a consideraciones operacionales lo más adecuadas tanto para la fisiología como para el rendimiento productivo de *P. taetrolens*. Así, la monitorización de la heterogeneidad fisiológica del sistema, a través de la citometría de flujo

multiparamétrica, ha proporcionado el conocimiento preciso sobre el comportamiento funcional de *P. taetrolens* asociado a la bio-producción de ácido lactobiónico. En el presente trabajo se ha avanzado, por lo tanto, en el conocimiento de cómo diferentes factores, tales como el pH operacional, la disponibilidad de oxígeno disuelto o la edad fisiológica, influyen decisivamente sobre el rendimiento, productividad y eficiencia del sistema productivo, constituyendo globalmente una valiosa información para una posible transferencia industrial del sistema aquí desarrollado.

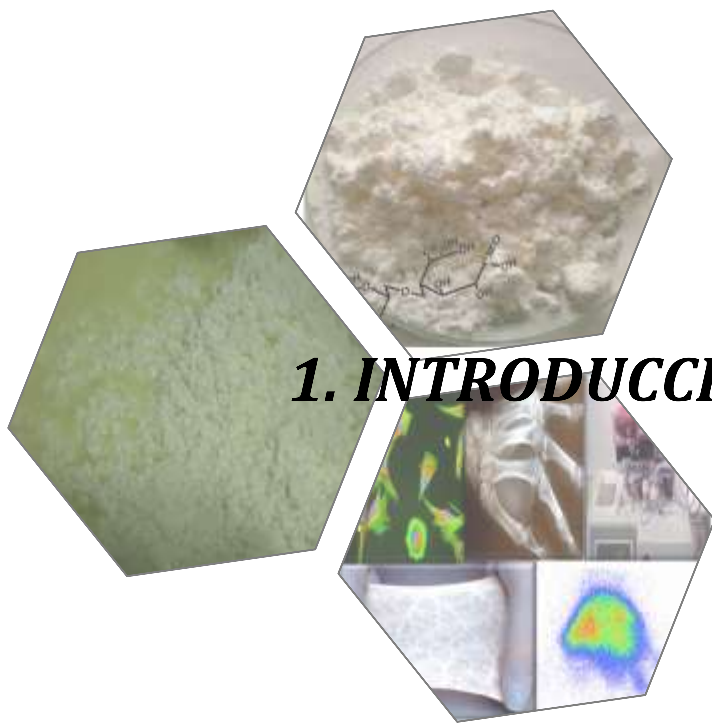
ABSTRACT

The recent growing commercial interest in the field of organic acids has stimulated their biotechnological production as the solution to the increasing demand generated on this chemical segment. Likewise, sustainability has been one of the challenges facing the biotechnology industry, which has been focused on both the development of efficient strategies and the biotechnological use of industrial wastes or byproducts. Within this context, this thesis has addressed the biotechnological production of two commercial organic acids such as lactic and lactobionic acid from two food wastes, cheese whey and yoghurt whey.

Firstly, it was studied the lactic acid production by *Lactobacillus casei* from fermentable sugars, which are available in the yoghurt whey removed from yoghurt derivates out of shelf life. Thus, the fermentative capacity of *L. casei* has been evaluated in terms of performance and efficiency within a fermentation featured by the presence of glucose, sucrose and lactose as substrates. In order to gain insight into the fermentative behaviour, multiparameter flow cytometry has been used as tool for the evaluation of cell functionality during the bioprocess. In fact, the application of this technique has enabled to know the cellular physiological status and system robustness at the population level.

Likewise, the bio-production lactobionic acid from whey by *Pseudomonas taetrolens* was studied under different bioprocessing conditions. Lactobionic acid has recently emerged into the market with a plethora of applications in areas such as food and biomedicine due to its excellent properties. To date, its industrial production is restricted to chemical catalysis, resulting in an inefficient and unsustainable methodology owing to costly catalysts as well as the formation of side-reaction products. The biotechnological production may overcome these drawbacks as the most appropriate alternative in order to meet the increasing demand of lactobionic acid. The bioprocess developed in the present study has been carried out at different scales in order to decipher the rate-limiting steps, optimizing the system based on appropriate operational considerations both for physiology and yield of *P. taetrolens*. Monitorization of the physiological heterogeneity through multiparameter flow cytometry has thus provided precise knowledge about the functional behaviour of *P. taetrolens* during the lactobionic acid bio-production. The present study has consequently added further knowledge on how different bioprocessing factors such as the operational pH, dissolved oxygen availability or physiological age may play a decisive role in the

fermentation performance, productivity and efficiency, representing valuable information for upscaling this system to the industrial sphere.



1. INTRODUCCIÓN

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1.1. Introducción

Los últimos años se han caracterizado por un indudable cambio de modelo productivo en la industria química, pasando de sistemas basados en fuentes no renovables hacia la implementación de procesos sostenibles y eficientes con una fuerte base biotecnológica. Dicho estímulo ha implicado el empleo de microorganismos como factoría celular para la producción de metabolitos de alto valor añadido, sirviendo como plataforma para la elaboración posterior de productos con indudable interés comercial. Así, el concepto de los microorganismos como factoría celular ha sido ampliamente estudiado desde el nacimiento de la microbiología industrial tras la segunda guerra mundial, siendo objeto de estudio tanto la producción microbiana de antibióticos, proteínas recombinantes o ácidos grasos como ejemplos más significativos.

La actual coyuntura caracterizada por las economías de escala y el imperante desarrollo económico de los países emergentes han incrementado sustancialmente la cantidad de residuos agroalimentarios generados a nivel mundial. Asimismo, la toma de conciencia medioambiental por parte del sector industrial y la implantación de medidas reguladoras ha espoleado la búsqueda de nuevas alternativas relativas al aprovechamiento de los residuos y subproductos generados. El tratamiento de estos residuos constituye una actividad que implica un importante coste económico y medioambiental para la industria. Así, se han venido mostrando esfuerzos en reducir la acumulación y generación de subproductos y residuos alimentarios. Sin embargo, el potencial para la reducción de los residuos es limitado y costoso. Bajo estas premisas se ha experimentado un notable desarrollo de nuevos procesos biotecnológicos donde se emplean estos residuos procedentes de actividades industriales, y carentes de valor comercial alguno, como sustratos con el fin de obtener productos de valor añadido. Estas nuevas bioconversiones microbianas no sólo juegan un indudable papel vertebrador de la sostenibilidad ambiental, sino que pueden ser utilizadas también con el fin de aportar nuevas y atractivas sustancias químicas emergentes con interés comercial.

Uno de estos subproductos industriales lo constituye el suero lácteo. Se trata de un subproducto que tradicionalmente se ha venido empleando únicamente como fertilizante o fuente de lactosa alimentaria. Múltiples han sido las aproximaciones realizadas con el fin de lograr el aprovechamiento y utilización de un residuo industrial como el suero lácteo. Así,

durante la última década, la utilización biotecnológica del suero lácteo ha surgido como una posible opción a la valorización del mismo, centrándose en la producción de diversos metabolitos microbianos como los ácidos carboxílicos, exopolisacáridos o más recientemente los polihidroxialcanoatos.

El procesamiento de leche en forma de yogures y productos líquidos fermentados ha seguido una tendencia claramente alcista en los últimos 10 años principalmente debido a la importante demanda experimentada en los mercados internacionales asociados a países emergentes. En concreto, el importante incremento del consumo asociado a la región asiática ha influido decisivamente en el incremento en la producción de más de 33 millones de toneladas en 2007 con respecto a 1997 (IDF, 2011; IDFA, 2008). Anualmente, el consumo de yogures y derivados se distingue por un apreciable incremento en todas las áreas comerciales, de hecho, el mayor aumento lo ha experimentado China, donde en 2007 la producción se situó en 2,5 millones de toneladas. La aparición de nuevos formatos de yogures que aportan beneficios extra, ha contribuido especialmente a que la tasa de crecimiento anual de estos productos se sitúe de media en un 6,2%, representando el 11% del mercado lácteo mundial (IDFA, 2008). En Europa, la elaboración de productos lácteos fermentados y de yogures contribuye con el 1% de los residuos generados por la industria alimentaria, mientras que el subsector lácteo fabricante de quesos en sus distintas variantes llega a generar el 26% de los residuos totales.

Actualmente, el empleo directo del suero lácteo se restringe a su utilización como complemento nutricional en piensos para animales, fertilizante o en último término se gestiona como efluente residual (Figura 1.1). Es en éste último contexto donde residen sus principales implicaciones medioambientales, debido a su notable potencial contaminante (175 veces superior al de un agua residual común) por su alta demanda química de oxígeno (DQO >60.000 ppm) y bioquímica de oxígeno (DBO >35.000 ppm), siendo considerado como el residuo más contaminante dentro de la industria alimentaria. Sin embargo, este subproducto contiene una importante cantidad de nutrientes que hacen atractivo su aprovechamiento biotecnológico con el propósito de obtener nuevos productos o derivados de lactosa que presentan un valor añadido superior y que también poseen nuevos nichos comerciales. De hecho, en la última década se han desarrollado nuevas tecnologías alrededor de técnicas cromatográficas, cristalización y membranas con el fin de aprovechar los principales componentes del lactosuero. Sin embargo, el notable incremento en volumen experimentado en el mercado tanto de lactosa de grado alimentario como de concentrados proteicos ha provocado una caída en los precios de los mismos. Esta excesiva oferta ha

implicado la búsqueda por parte de la industria de nuevos productos con un valor añadido superior y con un potencial de mercado más amplio.

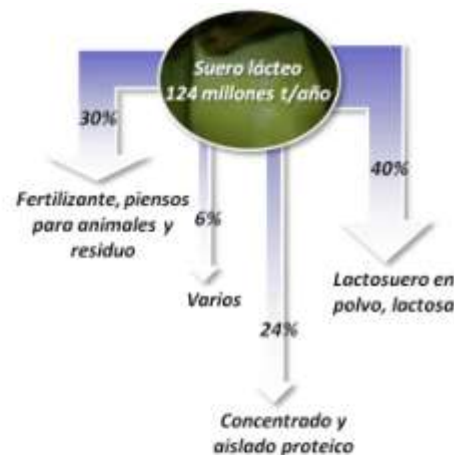

Figura 1.1. Utilización mundial del suero lácteo en 2006. De 177 millones de toneladas generadas durante 2006 sólo 124 (70% del suero lácteo generado) fueron utilizadas industrialmente como materia prima con el objeto de obtener productos derivados del suero lácteo o bien de la lactosa (datos obtenidos a partir de Affertsholt, 2007).

Por otra parte, la industria alimentaria está intentando adoptar el concepto holístico de producción alimentaria, integrando y actuando sobre todos los aspectos ambientales relativos al desarrollo y fabricación de un producto alimentario. Por lo tanto, la adopción de estos principios implica aportar un valor añadido en todos sus puntos a los residuos generados a lo largo de la cadena alimentaria. De esta forma, los productos lácteos fermentados no aptos para el consumo humano (incluyendo productos fuera de fecha de caducidad, cambios de lineal o sobre-stocks) tienen que ser retirados del mercado, representando un residuo para la industria láctea transformadora. De hecho, la industria láctea estima que alrededor del 1% de la producción total de productos fermentados lácteos tienen que ser tratados como residuos, con el consiguiente incremento en los costes de operación. La naturaleza de dichos residuos les confiere una alta potencialidad contaminante en base a su elevado contenido en azúcares añadidos. Además, se trata de productos cuyo reciclado tiene que ser tratado directamente por la industria y cuya posterior utilización se restringe únicamente al uso de la fracción sólida como pienso para animales. La fracción líquida resultante sí que puede ser aprovechada como fuente de carbohidratos fermentables en un proceso de fermentación ácido láctico. Los productos lácteos fermentados, caso del yogurt, se caracterizan por presentar altos contenidos en glucosa y sacarosa, azúcares que junto con la lactosa, constituyen una magnifica fuente de

carbono y energía aprovechable por bacterias ácido-lácticas como *L. casei*. Ésta bacteria es capaz de aprovechar estos carbohidratos mediante un proceso heteroláctico fermentativo facultativo para dar lugar a ácido láctico. Por consiguiente, el uso de este residuo en procesos fermentativos como sustrato reduce su potencialidad medioambiental, a la vez que se enfatiza en el concepto sostenible dentro de la cadena alimentaria y se genera un producto con un valor añadido superior.

La conversión oxidativa microbiana de lactosa presente en el suero lácteo puede significar una alternativa atractiva para la producción del ácido lactobiónico, un derivado de alto valor añadido que presenta importantes nichos de mercado. De hecho, este ácido orgánico presenta propiedades únicas que le confieren un amplio espectro de aplicaciones comerciales e industriales que van desde el ámbito cosmético al alimentario. La producción biotecnológica del ácido lactobiónico se espera que sea, por lo tanto, una alternativa industrial real que permita superar limitaciones como la formación de intermediarios o subproductos asociados al proceso de producción, algo que por ejemplo ocurre en muchos de los procesos oxidativos vía catálisis química.

1.2. *Objetivos*

El desarrollo de dos bioprocesos eficientes, efectivos, robustos y sostenibles para la obtención vía microbiana de ácido láctico y lactobiónico se ha convertido en el principal objetivo del presente trabajo. La presente memoria pretende aportar información relevante que contribuya a la implantación de la vía biotecnológica como la metodología de referencia para la producción industrial de ácido lactobiónico. En ambos bioprocesos se ha realizado un particular énfasis en la importancia de la fisiología microbiana a nivel poblacional como elemento clave en el desarrollo y optimización de los sistemas sometidos a estudio. Específicamente, se han planteado los siguientes objetivos:

- ✓ Evaluar la capacidad de la fracción líquida residual procedente de productos lácteos fermentados como fuente alternativa para la producción de ácido láctico.

- ✓ Conocer el comportamiento fermentativo de *L. casei* en una matriz natural y compleja basada en una mezcla de azúcares fermentables, estableciendo las condiciones operacionales más adecuadas.

- ✓ Caracterizar y monitorizar los diferentes estados fisiológicos de *L. casei* durante la producción fermentativa de ácido láctico.

- ✓ Desarrollar un sistema biotecnológico eficiente para la obtención de ácido lactobiónico por parte de *P. taetrolens*, estableciendo por lo tanto los parámetros operacionales más adecuados para maximizar la producción del mismo a partir de suero lácteo.

- ✓ Desarrollar protocolos de citometría de flujo multiparametrica como técnica que permita evaluar la funcionalidad de *P. taetrolens*.

- ✓ Conocer la imbricación del status fisiológico con la bioconversión oxidativa por parte de *P. taetrolens*. Revelar asimismo la heterogeneidad fisiológica inherente al sistema productivo en sus diferentes escalas de cultivo.

- ✓ Proponer las condiciones óptimas de operación a escala de biorreactor con el fin de alcanzar niveles industrialmente competitivos de ácido lactobiónico en un sistema de cultivo en discontinuo con alimentación.

1.3. Estructura de la memoria

La actual memoria se presenta como compendio de publicaciones que se enmarcan en la producción biotecnológica de los dos ácidos orgánicos que son objeto de estudio. Cada una de las publicaciones se ha elaborado con arreglo al esquema tradicional, esto es, dentro de cada artículo se puede distinguir específicamente una introducción, la descripción de la metodología empleada y una exposición crítica de los resultados obtenidos. Dichas publicaciones han sido aceptadas o están siendo evaluadas por revistas incluidas en el *Science Citation Index* (Thomson Reuters). La estructura de la presente memoria se encuentra dividida por lo tanto en ocho capítulos subdivididos cada uno de ellos en sus respectivos apartados.

En el Capítulo 1, correspondiente a la parte introductoria de la memoria, se exponen las causas reales que han motivado la realización del presente trabajo. Igualmente se presentan los objetivos que se han pretendido alcanzar. En el Capítulo 2 se describe el estado del arte en el que se encuentran los dos ácidos orgánicos sometidos a estudio en la presente memoria. En primer lugar, se introduce el contexto actual en el que se encuentra la producción vía fermentativa de determinadas sustancias dentro de la industria química. A continuación, se detalla someramente el estado en el que se encuentra la producción biotecnológica, así como las aplicaciones de un ácido orgánico maduro y ampliamente estudiado como es el ácido láctico. La tercera parte de las consideraciones teóricas se corresponde con un artículo conformado por una exhaustiva y documentada revisión bibliográfica sobre la bio-producción de ácido lactobiónico. En el mismo se puede encontrar tanto desde los orígenes del ácido, sus potenciales aplicaciones comerciales así como el status en el que se encuentra actualmente. En dicha revisión bibliográfica, el lector podrá advertir cómo el ácido lactobiónico ha llegado a convertirse, por ejemplo, en una sustancia emergente dentro de la industria cosmética debido a sus únicas propiedades terapéuticas.

En el Capítulo 3 se describen, de forma global, las diferentes metodologías experimentales utilizadas en la consecución de los resultados. Aunque la metodología específica de cada parámetro evaluado se detalla en su correspondiente artículo, en este capítulo se ha pretendido hacer hincapié en las técnicas comunes que han sido empleadas en la elaboración de las diferentes partes experimentales que componen la presente memoria.

Los subapartados del Capítulo 4 constituyen el bloque de resultados que conforma el eje central de la presente memoria. Cada subapartado se corresponde con su correspondiente artículo publicado o en vistas de serlo. Dichos trabajos son independientes entre sí, aunque

como se podrá comprobar fácilmente todos comparten un mismo elemento central. Se ha optado por comenzar con el aprovechamiento biotecnológico del suero de yogur (subapartado 4.1) y con la monitorización fisiológica de los bioprocesos implementados (subapartado 4.2). A continuación se exponen los restantes 5 artículos correspondientes a la producción biotecnológica del ácido lactobiónico. En dichos artículos se ha estudiado la influencia del tamaño de inóculo así como la descripción general del sistema a escala de matraz Erlenmeyer y biorreactor (subapartado 4.3), la disponibilidad de oxígeno disuelto (subapartado 4.4), la edad fisiológica (subapartado 4.5), el pH operacional (subapartado 4.6) y finalmente las estrategias de alimentación (subapartado 4.7).

En el Capítulo 5 se expone una discusión general sobre los aspectos más importantes hallados en la presente memoria, resaltando la dimensión y la relevancia de los parámetros operacionales que influyen sobre la producción microbiana de ambos ácidos orgánicos. Finalmente, en el Capítulo 6 se han condensado las conclusiones más importantes que se destilan del presente trabajo.

El Capítulo 7 está destinado a una bibliografía común a la memoria. Igualmente, se recogen algunos de los trabajos que deben de ser de obligada referencia para cualquiera que quiera conocer de primera mano las tendencias en microbiología industrial y abordar la producción microbiana de metabolitos de alto valor añadido.

La última parte de la memoria, bajo el titulo de Anexos, recoge la difusión de la tesis doctoral y el pertinente informe con el factor de impacto de las publicaciones.

2. CONSIDERACIONES TEÓRICAS

2. CONSIDERACIONES TEÓRICAS

2.1. Status actual de la producción biotecnológica de metabolitos microbianos

En los últimos años, la producción biotecnológica de determinados componentes químicos se ha convertido en la alternativa viable, sostenible y efectiva ante el continuo incremento en la demanda comercial mundial. Es indudable que la biotecnología ha jugado un papel fundamental desde el descubrimiento de la penicilina en 1927 por Fleming, momento que significó el nacimiento y posterior establecimiento de la fermentación industrial como metodología productiva de referencia. No obstante, la producción industrial de metabolitos microbianos por fermentación ha requerido el establecimiento de las bases científicas necesarias para lograr incrementar la producción, así como la búsqueda de nuevos organismos productores con características mejoradas (Demain, 2000, 2007). De hecho, la biotecnología industrial se ha convertido en un potente y floreciente sector económico con implicaciones directas en diversas industrias que van desde la automoción a la farmacéutica (Erickson et al., 2012; Hatti-Kaul et al., 2007). En 2030, se espera que un tercio de la producción mundial industrial esté relacionada con procesos biotecnológicos, presentando un volumen de mercado de más de 300 mil millones de dólares (Koln Paper, 2007). Asimismo, la OCDE también señala a la biotecnología como la herramienta clave en el desarrollo tanto de nuevas fuentes energéticas como de nuevos procesos industriales más sostenibles (OECD, 2007). Como resultado de tales perspectivas, en Europa se viene potenciando la denominada sociedad basada en el conocimiento biotecnológico, aglutinando las tendencias del mercado, perspectivas y las visiones globales dentro de la industria. En dicha alianza estratégica, que engloba varios programas marco, se ha fortalecido al sector biotecnológico europeo, encaramando a Europa como el segundo mercado en producción biotecnológica (EuropaBio y ESAB, 2006).

Los procesos biotecnológicos presentan la ventaja de ser sostenibles, reduciendo las emisiones atmosféricas de gases efecto invernadero, así como también muestran una reducida dependencia de los combustibles fósiles frente a los clásicos procesos químicos de producción (Hermann et al., 2007). El desarrollo de la biología de sistemas, la biología sintética o la ingeniería metabólica ha posibilitado asimismo la introducción de nuevas funcionalidades en los microorganismos (Curran y Alper, 2012), incrementando el portfolio

de bio-productos obtenidos mediante fermentación (Figura 2.1). Es indudable que la biotecnología industrial ha sufrido un enorme impulso gracias al nacimiento de la ingeniería metabólica hace ya más de 20 años por Jay Bailey (Bailey, 1991), una disciplina que ha permitido alcanzar el fenotipo microbiano deseado a través de la modificación del metabolismo celular (Curran y Alper, 2012; Yadav et al., 2012). Además, el desarrollo del concepto de biorefinería, donde las diferentes corrientes generadas durante la utilización de la biomasa son aprovechadas como materia prima, ha propiciado la irrupción de nuevos bio-productos fermentativos que o bien han reemplazado o están en vías de hacerlo a componentes químicos procedentes de combustibles fósiles.

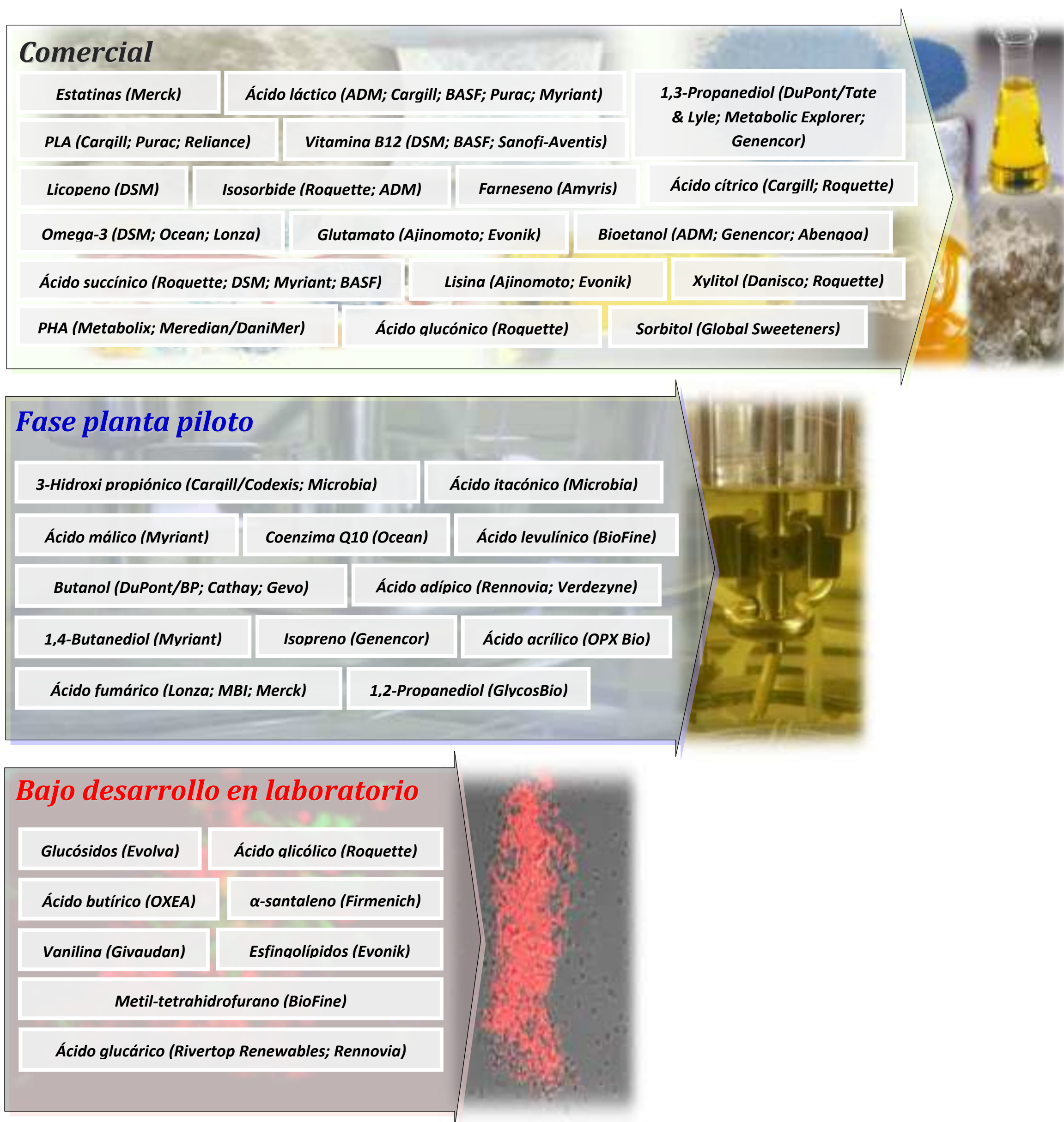

Figura 2.1. Portfolio de productos obtenidos por fermentación. Nótese que en la figura se obvian productos ampliamente consolidados tales como las enzimas, antibióticos y proteínas recombinantes, productos todos ellos obtenidos vía fermentativa a escala industrial.

En los últimos años han sido innumerables los lanzamientos al mercado de bio-productos obtenidos a partir de procesos fermentativos (Figura 2.1). De hecho, esta proliferación de bio-productos producidos vía fermentativa se ha correspondido con el desarrollo de un robusto sector conformado por multinacionales que han diversificado su portfolio de productos con el fin de sustituir muchos de los componentes químicos derivados del petróleo. De hecho, en el mercado se encuentran productos afianzados comercialmente como la lisina, el glutamato o el bioetanol. En el sector farmacológico son varios los bio-fármacos producidos por fermentación, caso de las estatinas producidas por Merck o la vitamina B12 por DSM (producida también con fines tecnológicos en el ámbito alimentario). Reseñar que muchos de los bioprocesos ya implantados a nivel industrial se basan en el empleo de materias primas renovables con bajo coste como residuos celulósicos o subproductos de diferentes industrias. Ejemplo en este sentido lo constituye la producción de farneseno por la norteamericana Amyris a partir de melazas de caña de azúcar o la misma obtención de polihidroxialcanoatos por Metabolix a partir de los mismos residuos. En fase de desarrollo a nivel de planta piloto se encuentran bio-productos tan atractivos como el butanol (resaltando la *joint-venture* conformada entre DuPont y BP para su desarrollo a nivel industrial), el ácido itacónico (en desarrollo por Microbia) o el isopreno (en desarrollo por Genencor). Igualmente, en incipiente estado de desarrollo se encuentran bio-productos tales como el ácido fumárico (en desarrollo por Lonza o Merck) o los glucósidos (caso de la producción microbiana de stevia, que se encuentra en fase de desarrollo por la compañía biotecnológica suiza Evolva) (Figura 2.1). Se estima que el mercado mundial de productos de fermentación generará en 2013 un volumen de negocio de 22000 millones de dólares dentro de un mercado altamente segmentado (Figura 2.2).

Particularizando dentro del sector químico, los ácidos orgánicos representan un segmento floreciente y con un importante crecimiento económico (Demain, 2007; Sauer et al., 2008). Se estima que el segmento químico conformado por los ácidos orgánicos producidos por fermentación va a experimentar un importate incremento en su volumen de negocio, pasando de los 2600 millones de dólares en 2009 a más de 4000 millones de dólares en 2013 (IEA, 2012). De hecho, se trata de un segmento dinámico conformado por ácidos maduros caso del cítrico, el láctico o el acético (reconocidos hoy en día casi como *commodities*) (Papagianni, 2011; Sauer et al., 2008) y por ácidos emergentes con un alto valor añadido que presentan nuevas y significativas aplicaciones gracias a sus singulares propiedades. Este último grupo está representado por ácidos como el glucárico, glicérico, fumárico, succínico, itacónico o el α-ceto glutárico, todos ellos en vista de ser producidos vía

fermentativa a nivel industrial (salvo el caso del succínico donde su producción biotecnológica ya está implementada por Reverdia).

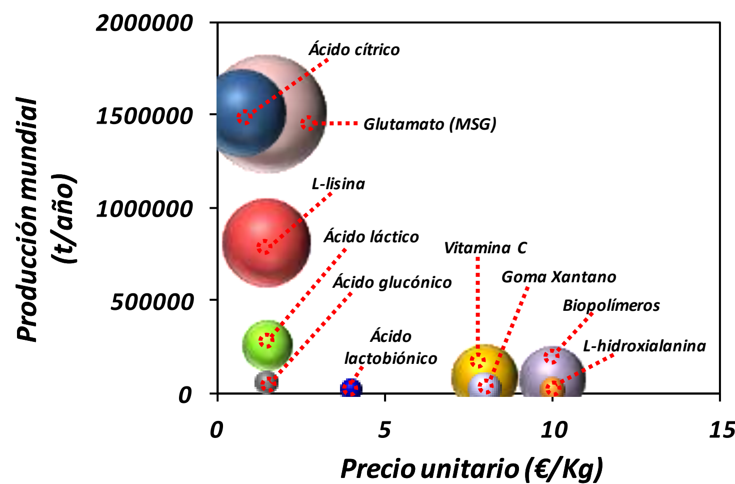

Figura 2.2. Mercado mundial de los principales bio-productos obtenidos por fermentación. El tamaño de la burbuja es proporcional al valor de mercado o volumen de negocio generado. En la gráfica se han excluido el bioetanol (con una producción mundial de 50 millones de t/año) y los antibióticos (35.000 t/año). Nótese que la presente figura ha sido elaborada en base a datos encontrados en diferentes fuentes bibliográficas (Demain, 2000, 2007; Sauer et al., 2008; Soetaert y Vandamme, 2010) que necesariamente no tienen que ser los valores reales, el lector tiene que poner en valor que la opacidad del mercado comercial sigue siendo todavía imperante. Los valores estimados correspondientes al ácido lactobiónico son datos referidos a su producción química puesto que las vías fermentativas o enzimáticas no han sido implementadas a nivel industrial.

La importancia de los ácidos orgánicos dentro del sector químico queda fuera de toda duda con el papel que se presupone que van a jugar dentro del futuro contexto químico industrial, cuyo ejemplo más clarividente en este sentido es la inclusión de hasta 9 ácidos orgánicos (succínico, fumárico, málico, 3-hidroxi propiónico, glucárico, aspártico, glutámico, itacónico y levulínico) dentro de los 15 futuros compuestos químicos con mayor potencial (Werpy y Petersen, 2004). El papel de los ácidos orgánicos emergentes dentro de la industria química se ha puesto asimismo de manifiesto con la transferencia e implementación industrial de nuevas plataformas microbianas. Así, por ejemplo, la francesa Roquette comercializa actualmente el ácido succínico producido por una *Escherichia coli* recombinante desarrollada en los laboratorios de los profesores George Bennett y Ka-Yiu San (Rice University, Houston, USA). De hecho, el sector industrial viene implementando tales avances como se ha puesto de manifiesto con la puesta en marcha de la primera planta industrial de producción fermentativa de ácido succínico por parte de Reverdia (*joint venture* entre DSM y Roquette), o la planta de producción de ácido succínico que Succinity

(*joint venture* entre BASF y CSM) pondrá en marcha a finales de 2013 en Barcelona con una capacidad de producción de 10.000 t/año.

Recientemente, un derivado del ácido succínico (en concreto el di-metil-succinato) ha sido objeto de acuerdo entre la belga Proviron y Reverdia, propiciando el desarrollo e implementación industrial de una nueva plataforma microbiana con objeto de producir biotecnológicamente di-metil-succinato que pueda reemplazar a los tradicionales polímeros derivados del petróleo. El año 2012 también ha sido clave en el nacimiento de alianzas empresariales con el fin de desarrollar nuevos sistemas fermentativos para producir ácidos orgánicos que hasta el momento sólo se producían vía química, tal es el caso de la alianza entre Novozymes, Cargill y BASF con el fin de producir ácido acrílico. Estos movimientos en este sector tan dinámico enfatizan el significativo papel que juegan actualmente los ácidos orgánicos dentro de la industria química.

2.2. *Producción biotecnológica de ácido láctico: status actual y aplicaciones*

El ácido láctico (ácido 2-hidroxipropanoico) representa a un ácido orgánico con un estado de desarrollo comercial maduro y cuya producción biotecnológica fue implementada a nivel industrial a principios de los años 90 del pasado siglo. El láctico es un ácido carboxílico que presenta un amplio espectro de aplicaciones y que hoy en día se obtiene industrialmente a través de procesos fermentativos llevados a cabo por bacterias ácido-lácticas tales como *Lactobacillus casei*, *L. helveticus* o *Lactococcus lactis*. Se trata del quinto producto fermentativo en volumen de producción (Figura 2.3), con una producción anual de 250.000 toneladas (Sauer et al., 2008). Actualmente es producido por compañías como Cargill o Purac a partir de fermentación de almidón de maíz.

Figura 2.3. Ranking mundial de productos obtenidos por fermentación. Datos tomados de Soetaert y Vandamme (2010).

El ácido láctico ha venido siendo empleado como conservante alimentario durante siglos, sin embargo, no fue hasta el descubrimiento de la fermentación por Pasteur en 1857 cuando se asoció que dicha actividad era provocada por microorganismos. En 1878, Joseph Lister aisló el primer cultivo puro de bacterias ácido-lácticas productoras de ácido láctico. Se trata de microorganismos microaerófilos Gram positivos que producen y secretan ácido láctico al medio. Además de las bacterias ácido-lácticas, el hongo *Rhizopus* es conocido como productor de ácido láctico. De hecho, en ocasiones es preferido como plataforma productora puesto que a diferencia de las bacterias ácido-lácticas, el hongo no requiere suplementación con nutrientes como el extracto de levadura o el licor de maíz fermentado. Además, *R. oryzae* sintetiza el isómero L(+) del ácido láctico, mientras la mayoría de las bacterias ácido-lácticas producen una mezcla racémica de ambos isómeros.

Las propiedades físico-químicas (véase Tabla 1) del ácido láctico le confieren una importante relevancia en diferentes aplicaciones industriales, tal y como se muestra en la Figura 2.4. Las aplicaciones de este ácido van desde el ámbito alimentario (empleado como agente aromatizante y conservante), cosmético (agente hidratante) al farmacéutico. No obstante, la producción de ácido láctico se espera que se vea significativamente incrementada ante la irrupción del ácido poli-láctico (PLA) como nuevo polímero biodegradable (Nampoothiri et al., 2010). Las perspectivas comerciales del PLA son halagüeñas como demuestra la *joint-venture* entre Cargill y Dow formando NatureWorks (propiedad actualmente en su totalidad de Cargill), la cual ya produce más de 150.000 toneladas anuales de éste biopolímero a partir de ácido láctico bajo la marca Ingeo. Otra relevante aplicación del ácido láctico en la industria química se encuentra asociada a la fabricación del solvente ecológico llamado etil-lactato.

Tabla 1. Propiedades y características del ácido láctico.

Propiedad	***Valores/características***
Naturaleza	Líquida
Fórmula molecular	$C_3H_6O_3$
Nombre sistemático	Ácido 2-hidroxi-propanoico
Peso molecular	90,08
pK_a	3,85
Punto de ebullición	122ºC
Solubilidad	Alta
Precio unitario	1,55 €/kg
Producción mundial anual (t)	250.000
Producción biotecnológica anual (t)	250.000
Método de producción	Fermentación
Principales fabricantes	Purac, Cargill, Galactic

Figura 2.4. Aplicaciones y usos comerciales en diversos campos del ácido láctico y/o sus sales.

En los últimos tiempos, los esfuerzos relacionados con la producción biotecnológica del ácido láctico se han centrado en tres ámbitos íntimamente interrelacionados, como son el desarrollo de nuevas plataformas microbianas productoras, el empleo de nuevas materias primas con bajo coste y la optimización del bioproceso (Figura 2.5). Estos dos últimos han sido los aspectos desarrollados en la presente memoria, tal y como se mostrará en los correspondientes subapartados (véanse subapartados 4.1 y 4.2).

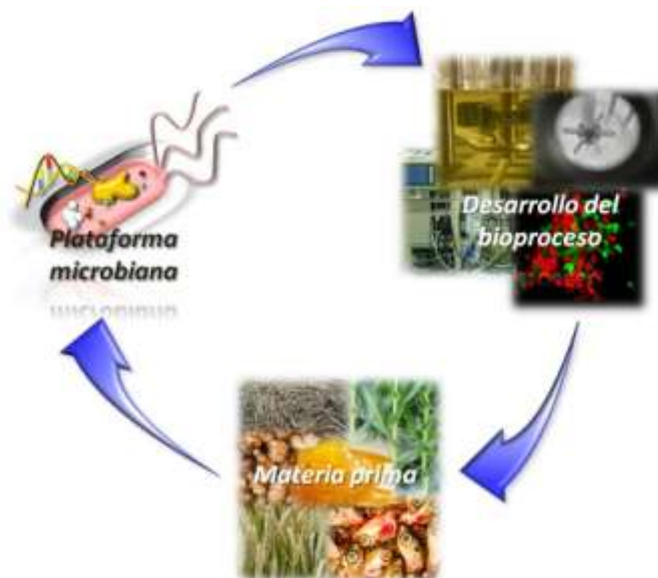

Figura 2.5. Pasos claves en la producción biotecnológica de ácido láctico.

En la producción biotecnológica de ácido láctico, se ha mostrado como papel fundamental la expresión de determinados genes involucrados en el metabolismo del carbono, el cual se regula y se controla en función de la información procedente del estado metabólico celular combinada con las señales externas que revelan la disponibilidad de determinados carbohidratos. De hecho, las fuentes de carbono más habituales en las fermentaciones ácido-lácticas lo representan sustratos con alta concentración en carbohidratos fermentables. De esta forma, en los últimos años los esfuerzos se han centrado en la utilización de residuos agro-industriales con altas concentraciones de carbohidratos en su composición. La atención se ha centrado asimismo en la optimización de procesos fermentativos teniendo como sustrato principal a materiales lignocelulósicos, los cuales pueden presentar altas cantidades de carbohidratos. Sin embargo, la utilización de estos materiales tiene el inconveniente de requerir pretratamientos previos a su utilización como sustrato, precisando de una hidrólisis previa (bien sea química o enzimáticamente) y/o una simultánea sacarificación de la celulosa y de la hemicelulosa.

Se estima que un 40-60% del coste total del proceso fermentativo lo representa el coste del sustrato o materia prima empleada, motivo por el cual se ha estimulado la búsqueda de nuevas materias primas renovables y con bajo coste que pudiesen ser utilizadas industrialmente. Diferentes materias primas procedentes de subproductos de origen alimentario han sido materia de estudio con el fin de ser sustrato fermentativo, tales como almidón, residuos agrícolas procedentes del cultivo de la cebada o el maíz, así como el propio salvado de arroz, la harina de trigo o el zumo de dátiles (Figura 2.6). Así, las últimas investigaciones van encaminadas en la sacarificación (degradación de la biomasa compleja) bien sea a través de hidrólisis ácida o enzimática y posterior fermentación (John et al., 2009). Ambas etapas pueden ser llevadas a cabo a través de una hidrólisis y fermentación o como sacarificación simultánea y posterior fermentación. De hecho, este proceso de fermentación está íntimamente ligado a la producción de ácido láctico a partir de materias con alto contenido en almidón (caso de la pulpa de mandioca, almidón de patata, salvado de harina o almidón de maíz) y de materiales celulósicos (tales como la celulosa o residuos madereros) utilizando microorganismos amilolíticos. No obstante, el empleo de esta metodología encarece los procesos de obtención de ácido láctico al requerir la hidrólisis previa de los sustratos. Recientemente, la fermentación de biomasa vegetal se ha centrado en el empleo de microorganismos como *L. amylovorus* que son capaces de realizar simultáneamente una primera sacarificación y posterior fermentación de los azúcares resultantes (John et al., 2009).

En adecuación al empleo de nuevos sustratos, se han venido igualmente desarrollando nuevas plataformas microbianas para la obtención de ácido láctico (Angermayr et al., 2012; Zhou et al., 2003). Estos esfuerzos, se han centrado últimamente en dotar a determinados microorganismos de nuevas funcionalidades, así como el desarrollo de cepas recombinantes más robustas y sin tantos requerimientos nutricionales. De hecho, se han conseguido cepas recombinantes de *E. coli* capaces de producir un nivel de ácido láctico cercano al rendimiento teórico (Zhou et al., 2003).

Figura 2.6. Materias y residuos empleados como sustrato en la producción biotecnológica de ácido láctico.

2.3. *Producción biotecnológica de ácido lactobiónico: status actual y aplicaciones*

En este subapartado relativo a las consideraciones teóricas se recogen la mayoría de los hallazgos o hitos referentes al ácido lactobiónico, así como los hechos acaecidos durante su más de un siglo de vida desde su descubrimiento por Fischer y Meyer en 1889. Se ha realizado una exhaustiva documentación acerca de la historia y cronología del ácido lactobiónico, enfatizando las actuales y potenciales aplicaciones de este ácido orgánico emergente. En la revisión se reflejan todos los avances relacionados con el ácido lactobiónico, que acompañados por una buena base documental permiten conocer las tendencias asociadas al empleo del ácido lactobiónico dentro de la industria cosmética, alimentaria o farmacológica. De hecho, se clasifica al ácido lactobiónico como una de las moléculas emergentes en el campo de la ingeniería tisular, la nanofarmacología o la biomedicina, circunstancia que no había sido advertida en ningún caso con anterioridad. Así, se ha puesto en valor por primera vez la relevancia y el potencial del ácido lactobiónico dentro de las disciplinas asociadas al ámbito biomédico, pasando desde la Solución Belzer al empleo de la tecnología *biónica* en la industria cosmética por NeoStrata. El incremento de la incidencia en el número de casos asociados al fallo hepático, que tras el cáncer es la 2ª causa de mortalidad en los países occidentales, lleva a inferir el relevante papel que jugarán en un futuro inmediato las terapias o metodologías que emplean al ácido lactobiónico como molécula necesaria no sólo para la correcta liberación controlada del fármaco de interés sino para la implantación exitosa de toda terapia celular hepática.

Se recoge igualmente el actual status del ácido dentro de la industria química o incluso su papel como aditivo alimentario. En definitiva, se ha reflejado el significado del ácido dentro de cada contexto comercial, enfatizando la importancia del ácido en las nuevas aplicaciones comerciales. Además, en esta revisión bibliográfica se describe la producción vía fermentativa y enzimática, subrayando por primera vez en la bibliografía las fortalezas y debilidades de cada uno de las metodologías.

Publicación: Bio-production of lactobionic acid: current status, applications and future prospects.

Situación: Enviada para su evaluación, bajo revisión.

Bio-production of lactobionic acid: current status, applications and future prospects

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Abstract

Lactobionic acid has appeared on the commercial scene as a versatile polyhydroxy acid with numerous promising applications in the food, medicine, pharmaceutical, cosmetics and chemical industries. This high value-added bio-product has recently received growing attention as a bioactive compound, providing an excellent chemical platform for the synthesis of novel potentially biocompatible and biodegradable drug delivery vehicles. Recent advances in tissue engineering and nanomedicine have also underlined the increased importance of this organic acid as a key biofunctionalization agent. The growing commercial relevance of lactobionic acid has therefore prompted the development of novel systems for its biotechnological production that are both sustainable and efficient. The present review explores recent advances and studies related to lactobionic acid bio-production, whether through microbial or enzymatic approaches, highlighting the key bioprocessing conditions for enhanced bio-production. Detailed overviews of the current microbial cell factories as well as downstream processing methodologies for lactobionic acid production are also presented. Furthermore, the potential prospects and current applications of this polyhydroxy acid are also discussed, with an emphasis on the role of lactobionic acid as a key platform in the development of novel drugs, biomaterials, nanoparticles and biopolymer systems.

Keywords: lactobionic acid; microbial fermentation; organic acids; bioprocessing conditions; bio-based production; drug delivery systems; galactosylated nanoparticles; galactosylated scaffolds.

1. Introduction

The production of bulk organic acid chemicals by microbial fermentation has undoubtedly undergone continuing growth over the last decade, progressively expanding its market niche and portfolio (Jang et al., 2012; Sauer et al., 2008). In fact, bacteria as bio-production platforms have become a reliable, cost-competitive, feasible alternative for large-scale industrial production of many bulk and specialty organic acids (Demain, 2007). Organic acids represent a growing chemical segment in which bio-based compounds such as fumaric, glyceric, propionic, xylonic, itaconic, levulinic or α-ketoglutaric acids have also emerged on the market as platform chemicals (Jang et al., 2012). This transition towards

bio-based industrial production has concomitantly involved the development of novel sustainable bioprocesses focused on the use of cost-effective renewable resources, either already implemented at an industrial level or still in the development pipeline (Willke and Vorlop, 2004). Beyond traditional organic acids, the market has also shown substantial interest in novel carboxylic acids like lactobionic acid due to its unique physicochemical properties (Fig. 1). Lactobionic acid (LBA) is a high value-added lactose derivative (Gänzle et al., 2008; Seki and Saito, 2012) which has recently emerged as a promising and versatile substance with countless applications in the cosmetics (Green et al., 2009; Tasic-Kostov et al., 2010; West, 2004a), pharmaceutical (Belzer et al., 1992) and food (Gerling, 1998; Gutiérrez et al., 2012) industries. The recent market glut suffered by traditional lactose-based products has additionally stimulated the dairy industry to seek new approaches for lactose utilization which could overcome the traditional view of lactose as a commodity (Affertsholt, 2007; Gänzle et al., 2008). As a result, novel lactose derivatives (such as lactitol, lactulose and LBA) have recently come onto the commercial market with considerable industrial applications (Playne and Crittenden, 2009; Seki and Saito, 2012).

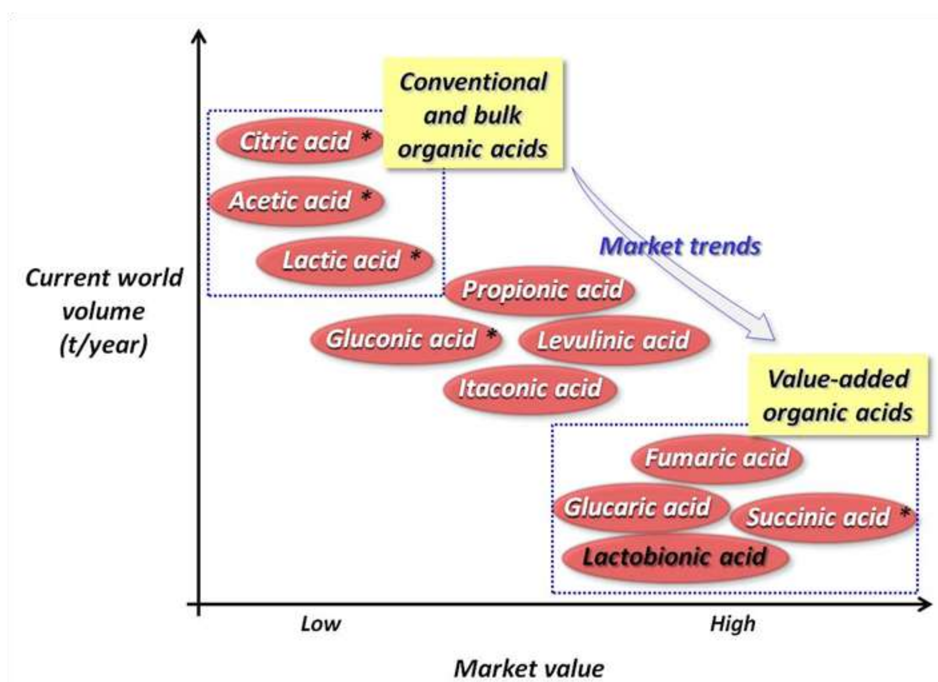


Figure 1. Current trends in the organic acids market. The market is moving from commodity bulk carboxylic acids towards value-added organic acids which display outstanding applications. LBA currently belongs to this latter segment. The symbol * denotes an implemented industrial production by microbial fermentation.

In recent years, LBA has also received growing attention as a bioactive molecule since it provides an excellent platform for the synthesis of biocompatible and biodegradable drug

delivery vehicles and biomaterials. In this respect, LBA will clearly play a major strategic role in the treatment of hepatic disorders through nanomedicine, with a potential near-term impact. Its prospect as a key biomolecule in the field of nanotechnology is thus of outstanding significance. In view of this commercial relevance, both the development and implementation of feasible LBA production systems emerge as crucial key challenges to meet market demands. To date, LBA is manufactured by chemical synthesis in an energy-intensive process which requires the use of costly metal catalysts (Kuusisto et al., 2008; Mirescu and Prübe, 2007; Yang and Montgomery, 2005). However, this expensive methodology may also involve the generation of undesirable side-reaction products (Chia et al., 2008). Although this polyhydroxy acid has been available since the late 1940s (Stodola and Lockwood, 1947; Stodola and Jackson, 1950), its production by biotechnological means has not been developed so intensively up to now in comparison with other organic acids such as lactic, succinic or citric acid (Papagianni, 2011). Nevertheless, bio-production of LBA has emerged as both a promising and feasible approach to meet the growing demand for this bio-product. Furthermore, environmentally-friendly and cost-effective LBA bio-production can be accomplished by employing cheese whey as an inexpensive feedstock (Alonso et al., 2011, 2012a). Despite being a traditional natural source for whey protein isolate (Smithers, 2008) and lactose (Schaafsma, 2008), cheese whey upgrading and treatment remain as two of the major challenges facing the dairy industry. Therefore, the search for innovative solutions in the disposal and management of this high-strength waste stream has become the driving force behind the development of novel sustainable biotechnological processes (Guimarães et al., 2010).

Within this context, the present review explores recent advances in LBA bio-production, either through enzymatic or microbial biosynthesis, as well as the current novel trends addressing the application of LBA in the marketplace, with particular emphasis on those emerging areas such as nanomedicine and tissue engineering. A detailed overview of current microbial cell factories, further downstream processing methodologies for LBA production and prospects are also provided.

2. Properties and current industrial status of lactobionic acid.

The structure and physicochemical properties of LBA confer on it a plethora of current and potential commercial applications, as shown in Figure 2. This organic acid exhibits a large number of newly discovered biological activities and great therapeutic potential due to its excellent biocompatibility, biodegradability and nontoxicity, as well as its chelating, amphiphilic and antioxidant properties. LBA belongs to the aldobionic family of acids, which

additionally comprises maltobionic and cellobionic acid (Pezzotti and Therisod, 2006). From a nutritional point of view, this substance may be considered a low-calorie sweetener which provides only 2 kcal/g (Schaafsma, 2008). Chemically, it comprises one galactose molecule attached to one molecule of gluconic acid via an ether-like linkage (its molecular structure is shown in Table 1). In particular, the presence of multifunctional groups (8 hydroxyl groups) converts LBA molecule into a metal ion chelator which can sequester cations such as calcium (Abbadi et al., 1999). This carboxylic acid presents a molecular weight of 358.3, with a pKa of 3.6 (Armarego and Chai, 2009). Table 1 lists the main physicochemical properties of LBA; its high water solubility being especially noteworthy. The calcium salt of LBA in fact shows 40,000 times higher water solubility than calcium carbonate and 10 times that of calcium lactate (Nakano et al., 2010).

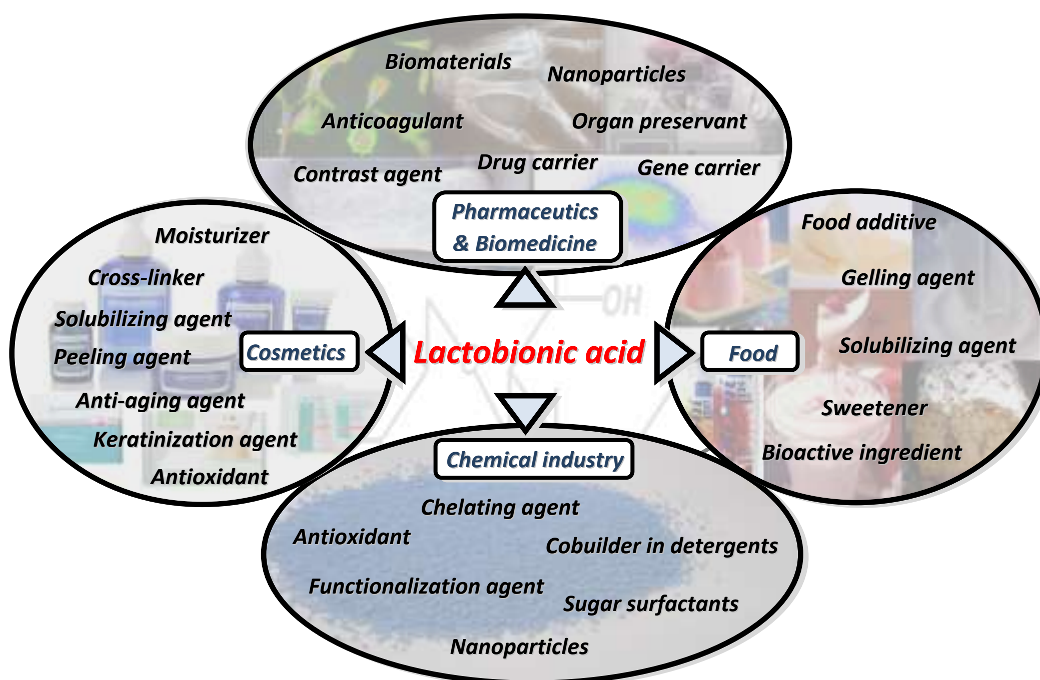
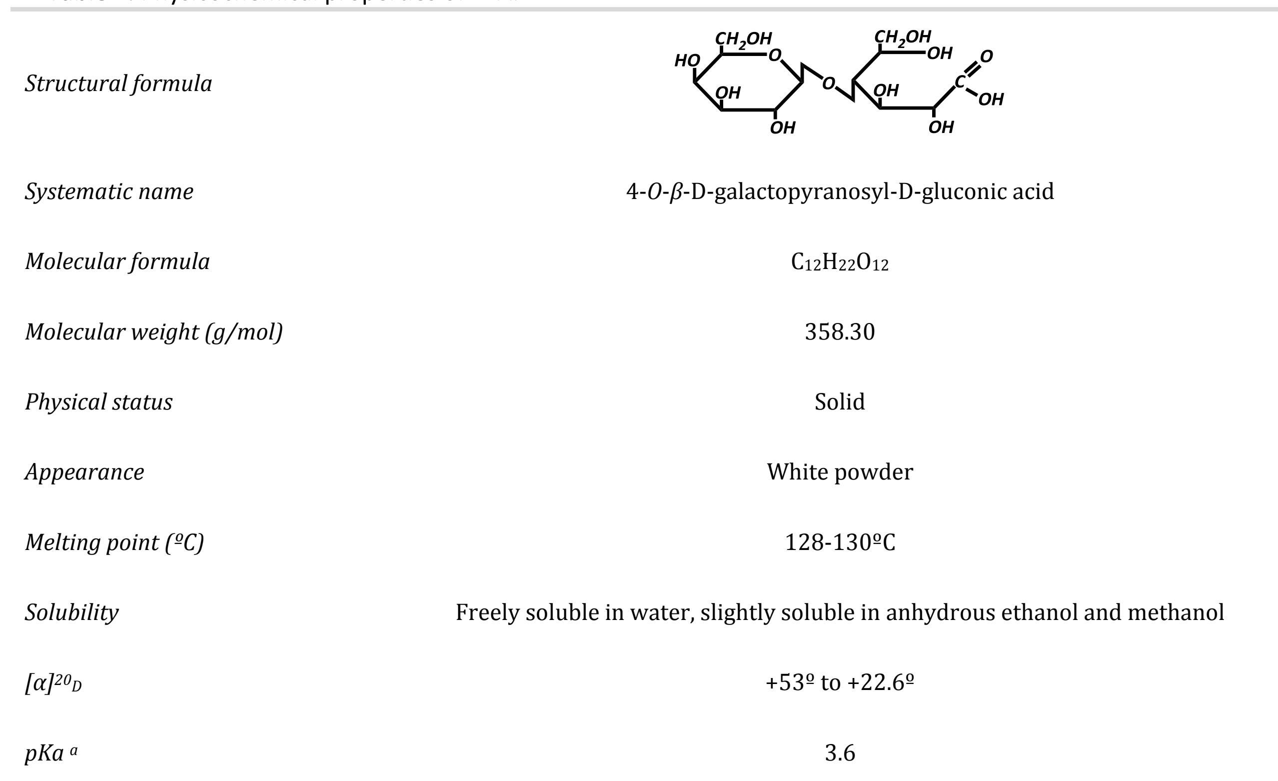


Figure 2. Overview of LBA applications.

LBA was reported and synthesized for the first time by Fischer and Meyer (1889) as the oxidation product of the lactose free aldehyde group after a chemical oxidation with Bromine. In the industrial manufacturing context, LBA is currently produced by chemical synthesis from refined lactose in an energy-intensive process (Kuusisto et al., 2007; Yang and Montgomery, 2005). This manufacturing process also involves the use of harmful and costly catalysts, as well as the generation of undesirable side-reaction products (Murzina et al., 2008). Aside from bio-production methods, catalytic wet oxidation and electrochemical catalysis of lactose have also been proposed as methodologies to obtain LBA. However,

these systems involve costly operational processes (Drulliole et al., 1995; Magariello, 1956) or the generation of by-products during the course of the reaction (Chia et al., 2008). Likewise, increasing environmental requirements will undoubtedly drive LBA industrial production towards more sustainable production systems that, to date, otherwise not complied with chemical catalytic oxidation or enzymatic methods.

Table 1. Physicochemical properties of LBA.



Structural formula	
Systematic name	4-*O*-*β*-D-galactopyranosyl-D-gluconic acid
Molecular formula	$C_{12}H_{22}O_{12}$
Molecular weight (g/mol)	358.30
Physical status	Solid
Appearance	White powder
Melting point (ºC)	128-130ºC
Solubility	Freely soluble in water, slightly soluble in anhydrous ethanol and methanol
$[\alpha]^{20}_{D}$	+53º to +22.6º
pKa [a]	3.6

[a]: 25ºC

The major industrial manufacturers of LBA are Solvay (Germany), FrieslandCampina Domo (Netherlands), Sandoz (Germany), Reliable Biopharmaceutical Corporation (USA) and the US Dairy Ingredient Company (USA) (Affertsholt, 2007; Playne and Crittenden, 2009). Although no official data are available on its annual world production (Playne and Crittenden, 2009), LBA has burst onto the commercial scene as a prominent emerging substance with a significant improved commercial rate in recent years (Affertsholt, 2007). In view of LBA market forecasts, Solvay in fact opened a plant in Germany in the late 1990s with the aim of achieving an annual production of 1000 tons (Gerling, 1998).

3. Use and commercial applications of lactobionic acid.

3.1 Health and biomedical applications: from drug-delivery systems to preservative solutions

3.1.1 Drug-delivery systems

Recent trends in biomedicine have witnessed the appearance of novel techniques and strategies dealing with nanotechnology, tissue engineering, drug-delivery systems or biomaterials based on biodegradable and biocompatible chemicals which are coming increasingly to the fore. Targeted delivery of therapeutic agents has thus emerged as a promising approach in medicine not only due to its increased therapeutic efficacy, but also to its lesser side effects. In fact, the lack of effective and practical pharmacological approaches for targeting liver cells has become the driving force behind the development of effective drug-delivery systems employing LBA as a ligand. In this context, LBA offers unique properties such as biocompatibility, biodegradability, ion-chelating ability and self-assembly, in addition to their synergistic combination, which confer on LBA a plethora of applications as a vehicle for the innovative treatment of many potential diseases. Owing to these unique properties, LBA provides an excellent platform for the synthesis of potentially biocompatible and targetable drug delivery vehicles, from DNA to bioactive molecules. Table 2 lists recent research studies that have focused on the development and assessment of drug-delivery systems containing LBA as a ligand.

The current growing prevalence of hepatic disorders has become the driving force behind the development of novel therapeutic treatments based on hepatic-targeted drug delivery systems (Chen et al., 2012a). Targeting hepatoma cells constitutes a promising approach for clinical chemotherapy of liver cancer which is currently a subject of intensive research efforts (Chen et al., 2012b; Duan et al., 2011; Huang et al., 2011; Na et al., 2000; Varshosaz et al., 2012).

LBA-based drug delivery systems can successfully target hepatocytes due to the presence of asialoglycoprotein receptors (ASGPR) located on their surface, since LBA works as a ligand of these receptors (Kim et al., 2006; Peng et al., 2007). These receptors represent the preferential targets for potential hepatic interventions, as the therapeutic effect of any drug is often limited by the difficulty of accessing specific sites. This receptor additionally provides a membrane-bound active site for cell-to-cell interactions (Stockert, 1995). Considerable emphasis has been placed on the surface modification of polysaccharides by galactosylated groups from LBA targeting ASGPR. The functionalization of LBA may thus achieve enhanced binding to the cell surface on hepatocytes (Park et al., 2001). Furthermore, successful receptor-mediated cell transfection in targeted specific tissues such

as epithelial cells has been achieved through the functionalization of PEI and PEI-PEG-copolymers with LBA (Weiss et al, 2006).

Table 2. Comparison of research studies using LBA as the target ligand for drug delivery purposes.

System	*Applications*	*Polysaccharide*	*Drug*	*Graft material/ polymer*	*Targeted cells*	*Reference*
Nanoparticles	Drug delivery	Chitosan	Oridonin	n.u.	Hepatocytes	Zheng et al. (2012)
Nanoparticles	Drug delivery	Chitosan	Doxorubicin	n.u.	HepG2	Jain and Jain (2010)
Nanoparticles	Drug delivery	Chitosan	n.u	n.u	HepG2	Mi et al. (2007)
Nanoparticles	PET imaging	Chitosan	Fluorine-18	n.u.	Hepatocytes	Yang et al. (2010)
Nanoparticles	Drug delivery	Chitosan	n.u	MPEG	Hepatocytes	Lin et al. (2009)
Core-shell polymeric nanoparticles	Drug delivery	Biotinylated poly(ethylene glycol)	ATRA	n.u.	Hepatocytes	Kim and Kim (2003)
Core-shell polymeric nanoparticles	Drug delivery	Diamine-terminated poly(ethylene glycol)	Clonazepam	n.u.	Hepatocytes	Kim and Kim (2002)
Microcapsules	Drug delivery	Chitosan	n.u.	n.u.	Hepatocytes	Zhang et al. (2011)
Gene carrier	DNA delivery	Dextran	n.u.	PEI, PEG	Hepatocytes	Weiss et al. (2006)
Gene carrier	DNA delivery	Chitosan	n.u.	Spermine	HepG2	Alex et al. (2011)
Gene carrier	DNA delivery	PEG	n.u.	Poly-L-lysine	HepG2	Choi et al. (1998)
Gene carrier	DNA delivery	Chitosan	n.u.	n.u.	HepG2	Gao et al. (2003)
Gene carrier	DNA delivery	Chitosan	n.u	n.u	HepG2	Lin et al. (2011)
Gene carrier	DNA delivery	Chitosan	n.u	Dextran	Hepatocytes	Park et al. (2000)
Gene carrier	DNA delivery	Chitosan	n.u.	PEG	Hepatocytes	Park et al. (2001)
Gene carrier	DNA delivery	Chitosan	n.u.	PVP	Hepatocytes	Park et al. (2004)
Gene carrier	DNA delivery	Chitosan	n.u.	n.u.	HepG2	Kim et al. (2004)
Gene carrier	DNA delivery	PEI	n.u.	PEG	HepG2	Kim et al. (2005a)
Gene carrier	DNA delivery	Chitosan	n.u.	PEI	HepG2	Kim et al. (2005b)
Gene carrier	DNA delivery	Chitosan	n.u.	PEI	Hepatocytes	Jiang et al. (2007)
Gene carrier	DNA delivery	Chitosan	n.u.	PEG	Hepatocytes	Jiang et al. (2008)

Table 2. (Continued)

System	*Applications*	*Polysaccharide*	*Drug*	*Graft material/ polymer*	*Targeted cells*	*Reference*
Drug carrier	Photodynamic therapy	n.u.	Porphyrin	n.u.	Hepatocytes	Li et al. (2007)
Liposomes	DNA delivery	Poly-L-lysine	n.u.	n.u	HepG2	Wang et al. (2006a)
Liposomes	Drug delivery	n.u.	Doxorubicin	n.u.	Hepatocytes	Wang et al. (2006b)
LDL nanoparticles [a]	Antigen delivery	n.u.	n.u.	n.u.	Macrophages	Wu et al. (2009b)
Micelles	Drug delivery	PEO-PPO-PEO	Silybin	n.u.	Hepatocytes	Li et al. (2009)
Micelles	Drug delivery	Polycaprolactone	Prednisone	Dextran	HepG2	Wu et al. (2009a)
Micelles	Drug delivery	PEG	Paclitaxel	Poly(Ɛ-caprolactone)	Hepatocytes	Yang et al. (2011)
Micelles	Photodynamic therapy	Polycaprolactone	Porphyrin	n.u.	HepG2	Wu et al. (2010)

PET: positron emission tomography; PEG: poly(ethylene glycol); PVP: Poly(vinyl pyrrolidone); PEI: polyethylenimine; HepG2: liver hepatocellular cells; ATRA: anti-cancer drug all-*trans*-retinoic acid; PEO-PPO-PEO: poly (ethylene oxide)-co-poly(propylene oxide)-co-poly(ethylene oxide) (Pluronic triblock copolymer); MPEG: methoxy poly(ethylene glycol).
n.u.: not used
[a] Conjugated with fluorescein ovalbumin.

Recent developments in nanotechnology have also raised a great deal of interest in the design and formulation of targeted non-viral delivery constructs for siRNA *in vivo* delivery capable of overcoming barriers. In this regard, chemical modification of chitosan for the delivery of DNA and siRNA has become a valuable tool for treating acquired diseases through the transfection of malign cells using gene therapy (Alex et al., 2011). A hepatocellular carcinoma cell-targeted gene carrier has also been approached by means of poly-L-lysine grafted with LBA and poly(ethylene glycol) (Choi et al., 1998), low molecular weight chitosan (Gao et al., 2003; Ill'ina and Varlamov et al., 2007) and chitosan-graftpolyethylenimine (Jiang et al., 2007). LBA has also been employed for the specific targeting of a rotavirus capsid VP6 to hepatocytes or hepatoma cells bearing ASGPR receptors, achieving increased enhancement of the receptor-mediated endocytosis in hepatoma cells (Zhao et al., 2011). Potential protection from fulminant hepatitis was achieved by Dong et al. (2009) through targeting delivery of antisense oligonucleotides containing LBA into Kupfer cells with the aim of reducing the expression of liver tumour necrosis factor-α. In addition, complexes of DNA/polyethylenimine-graft-poly(ethylene glycol) (Kim et al., 2005a) or poly(ethylenimine) (Kim et al., 2005b) have been shown to be efficient gene carriers. LBA has been also successfully introduced into water-soluble chitosan through an amide linkage in order to improve not only the hepatocyte specificity of

DNA carriers, but also the transfection efficiency of the complexes into hepatoma cells (Kim et al., 2004). The transfection efficiency of DNA into hepatoma cells has been also enhanced in synthesized PEGylated chitosan polyplexes (<200 nm) bearing LBA (Lin et al., 2011).

Chitosan nanoparticles functionalized with LBA have been proposed as alternative and biocompatible cationic polymers for non-viral gene therapy (Jiang et al. 2008). Nanoparticles comprising galactosylated chitosan (Kim and Kim, 2002; Kim et al., 2004; Kim et al., 2005b), galactosylated poly(ethylene glycol) (Kim and Kim, 2003), galactosylated polyethylenimine-*graft*-poly(ethylene glycol) (Jiang et al., 2008), galactosylated poly(ethylene glycol)-chitosan-*graft*-polyethylenimine (Kim et al., 2005a) or galactosyl conjugated N-succinyl-chitosan-graft-polyethylenimine (Lu et al., 2010) have likewise been used as gene carriers with enhanced transfection efficiency and hepatocyte specificity both *in vitro* and *in vivo* due to the presence of LBA as functional group (Table 2).

LBA can also serve as a chemical platform for the biosynthesis of antioxidant carriers (Ortial et al., 2006). Amphiphilic copolymeric sub-micro particles containing LBA have recently been devised as a carrier shielding from enzymatic attack for peptide and protein delivery (Meng et al., 2012). Tian et al. (2008) have reported the synthesis, self-assembly and *in vitro* evaluation of a novel amphiphilic glycopeptide triblock copolymer (polytetrahydrofuran PTHF, poly(L-lysine)s PLLs and *ε*-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride) containing LBA as a carrier for controlled drug release.

In addition to the above drug delivery systems containing LBA as a ligand molecule, a novel and promising controlled-release system has also been recently devised. Interestingly, Luo et al. (2011, 2012) have developed a biocompatible controlled-release system based on mesoporous silica nanoparticles for intracellular pH-responsive delivery of anticancer drugs using LBA (conjugated to BSA) as an end-cap. These authors devised an efficient nanoreservoir for efficient intracellular anticancer drug delivery with high cell-specific endocytosis and intracellular pH-responsive controlled drug release which induces effective cell apoptosis. These nanoparticles may thus not only serve as intelligent nanoreservoirs of drugs, but also control anticancer drug release in response to intracellular acidic pH (Luo et al., 2011, 2012). Besides, pH-responsive nanogels using LBA as active targeting has been employed for hepatoma-targeted delivery of oridonin, exhibiting a higher antitumor activity than drug-loaded nanogels without LBA (Duan et al., 2011). A potential liver-targeting prolonged-circulation polymeric prodrug of doxorubicin containing LBA was developed likewise by Huang et al. (2010). This novel antitumor system showed better *in vivo* antitumor efficacy and lower cytotoxicity, suggesting its great potential as highly efficient polymeric antitumor prodrug (Huang et al., 2010).

3.1.2 Nanoparticle diagnosis

Recent advances in the synthesis and surface modification of nanoparticles using LBA have emerged as promising candidates for novel diagnostic tools in biomedicine. Bio-functionalization agents such as LBA have attracted increasing attention in recent years as surface coating materials due to their enhanced properties. Specifically, bio-functionalized nanoparticles exhibit several key advantages compared to traditional therapeutic tools, such as enhanced biodegradability, biocompatibility, detection sensitivity, water-solubility, longer stability and lower cytotoxicity (Knopp et al., 2009). As a result, these nanoparticles are used for biomedical applications such as bio-detection, cancer therapies, magnetic resonance imaging or bio-labelling. Table 3 summarizes the recent research studies on nanoparticles containing LBA as a coating material.

Table 3. Summary of research studies dealing with nanoparticles containing LBA as a coating material.

Nature of nanoparticles	*Synthesis method*	*Applications*	*Coating effect*	*Average size (nm)*	*Targeted cells*	*Reference*
Manganese oxide surface doped gadolinium oxide (Gd_2O_3@MnO)	Synthesis + doping	MRI contrast agent, liver diagnosis	Improved biocompatibility and water solubility	4.6	Kidney	Choi et al. (2010)
Magnetic iron oxide	Co-precipitation	Clinical diagnosis	Improved stability and dispersibility	8-10	Hepatocytes	Bahadur et al. (2009)
Magnetic iron oxide	Co-precipitation	Liver diagnosis	Improved stability and increased cellular internalization	26	Hepatocytes	Selim et al. (2007)
Cadmium sulfite	Arrested precipitation	Liver diagnosis	Improved biocompatibility and increased cellular internalization	35	Hepatocytes	Selim et al. (2009)
Magnetic iron oxide	Co-precipitation	Liver diagnosis	Improved stability	120	Hepatocytes	Kekkonen et al. (2009)

MRI: Magnetic Resonance Imaging.

Surface coating of nanoparticles with LBA contributes to enhanced physicochemical stability, as well as increased uptake by hepatocytes. Nanoparticles combining LBA (its galactose moiety) and diamine-terminated poly(ethylene glycol) exhibited *in vitro* release of A1 1-trans-retinoic acid over 1 month, underlining the key role of LBA as a surface coating material (Kim and Kim, 2003).

In view of the increasing demand for novel magnetic resonance imaging contrast agents, numerous approaches have employed LBA-functionalized nanoparticles for bioimaging applications. These novel bioimaging techniques play a vital role in diagnosing diseases,

providing enhanced sensitivity and advanced functionalities (Kekkonen et al., 2009; Selim et al., 2007; Richard et al., 2009). As a result, LBA conjugated to amino-modified silica (fluorescent) nanoparticles has been shown to be an efficient and promising probe in the detection and identification of liver cancer cells in cellular complex matrices such as blood (Peng et al., 2007).

Another recently developed approach to targeting liver cancer cells involved the use of synthesized glycoconjugated poly(amindoamine) dendrimers as diagnostic tools (Guo et al., 2012). These dendrimers were synthesized by conjugating the fluorophore fluorescein isothiocyanate and LBA to confirm their *in vitro* targeting capabilities as key elements for the development of efficient strategies targeting carcinoma cell lines (Guo et al., 2012).

3.1.3 Tissue engineering

More recently, tissue engineering has emerged as a promising area for the development of novel functional replacement tissues (scaffolds and biomaterials) aiming to substitute, repair or regenerate injured tissues and organs. Liver tissue engineering using hepatocytes and scaffolds has been developed as an alternative method for the treatment of liver failure. In view of the growing incidence of hepatic disorders, new approaches are needed not only to meet the liver shortage problem, but also to provide innovative solutions for regenerative medicine (Kim et al., 2008). Although LBA does not contribute directly to the growth of any tissue or biomaterial, this organic acid may facilitate the attachment and entrapment of hepatocytes, as well as the establishment and construction of biocompatible scaffolds for liver tissue engineering purposes. Table 4 summarizes the noteworthy recent research studies focussing on the use of LBA as a bio-functionalization agent in biomaterials or biocompatible scaffolds for tissue engineering.

Scaffolds generated from natural polymers such as alginates, chitosan or glycosaminoglycans, as well as from synthetic polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) or poly(caprolactone) (PCL), have been widely explored as tissue engineering materials. However, their functional capacities and biocompatibility may be enhanced through bio-functionalization with LBA. Novel lactone-based glycopolymers, galactosylated chitosan (Fan et al., 2010), polycaprolactone scaffolds modified with galactosylated chitosan (Qiu et al., 2012) and alginate/galactosylated chitosan sponge for liver-tissue engineering have thus been developed in the last decade (Yang et al., 2001). Specifically, the incorporation of functional groups such galactose moieties in the polymer backbone enables the improvement of its functional properties, exhibiting higher levels of liver-specific function maintenance in terms of albumin secretion or urea synthesis (Choi et

al., 2012; Feng et al., 2009). The interaction between ASGPRs and the galactose ligands displayed by LBA is fundamental to enhance hepatocyte adhesion, hepatocyte aggregation, maintenance of liver-specific functions and mechanical stability (Kim et al., 2011). In fact, LBA-based scaffolds may be better recognized by hepatocytes through ASGPR receptors, showing better cell viability and long-term maintenance of liver-specific functions (Feng et al., 2009).

Table 4. Summary of noteworthy recent research studies on biomaterials using LBA.

Scaffold/polysaccharide	*Applications*	*Conformation*	*Reference*
Polycaprolactone	Hepatocyte attachment	Film	Qiu et al. (2012)
Alginate/chitosan	Hepatocyte attachment	Porous	Yang et al. (2001)
Alginate	Hepatocyte attachment	Film	Chung et al. (2002)
Alginate	Hepatocyte entrapment	Film	Yang et al. (2002)
Chitosan	Hepatocyte aggregation	Nanofibrous	Feng et al. (2009)
Chitosan	Hepatocyte culture	Film	Fan et al. (2010)

The design of liver tissue-mimicking microenvironments with bioactive scaffolds constitutes a key challenge for the field of tissue engineering. Without a doubt, synthetically engineered matrices for cellular recognition and attachment constitute pivotal elements for the success of any tissue engineering approach. Artificial extracellular matrices may play a key role in tissue engineering since they provide cell-adhesive substrate, control of three-dimensional tissue structure, and the presentation of growth factors or cell-adhesion signals (Kim et al., 2011). In fact, galactosylated-matrices through LBA have been proved to be effective in hepatocyte entrapment and attachment. In this regard, a LBA-functionalized chitosan has been described as an effective extracellular matrix for hepatocyte attachment (Mi et al., 2006; Park et al., 2003). Specifically, the incorporation of LBA in a synthetic polymer such as poly(vinyl alcohol) may provide a better microenvironment for cell aggregation along with nutrition and metabolite transfer (Guo et al., 2003). Photosensitive poly(allylamine) containing LBA has been also suggested as effective extracellular matrix (Kang et al., 2004; 2005). A biological adhesive comprising a photocrosslinkable chitosan molecule formed by LBA and a photoreactive azide group has likewise been developed as an innovative solution for hepatocyte attachment, which facilitates cell-cell as well as cell-

matrix interactions (Ono et al., 2000). Furthermore, a novel thermo-responsive biomaterial containing LBA has been recently devised for biomedical and tissue engineering by Wang et al. (2010). This synthesized copolymer was not only capable of improving the cellular viability but also retained the activity of the detached cells (Wang et al., 2010).

3.1.4 Other health-related products

In addition to the aforementioned applications, LBA is already being widely used in health-related products such as pharmaceuticals, antibiotics and preservative solutions for organ transplantation. This polyhydroxy bionic acid has been used as an adjuvant in the preparation of erythromycin for treating bacteremia (Hirakata et al., 1992) and in formulations containing chlorhexidine as an antiseptic substance (Werle et al., 2002). LBA may also work as a stabilizer of pharmaceutical formulations containing antibodies (Agarkhed et al., 2010) or interferons (Goldstein and Thatcher, 1990), and as a solubilizer to enhance the aqueous solubility of argatroban, an antithrombotic agent, in alcohol-free pharmaceutical compositions (Palepu, 2008). Furthermore, novel potential applications have emerged in recent years, such as the use of LBA-based derivatives to prevent and treat malaria (Agustí et al., 2004; Giorgi et al., 2010). More recently, an ophthalmic composition based on LBA as a therapeutic agent has been devised for the therapeutic treatment of corneal edema and inflammation, overcoming the drawbacks displayed by conventional hyperosmotic topical formulations (Cavallo et al., 2012). Stannard et al. (2010) have demonstrated that LBA may act as a specific high affinity inhibitor compound of carbohydrate binding proteins (galectin-blocking), which promotes vaccine-stimulated immune responses against *in vivo* breast tumours that may lead to a decrease in tumour progression, as well as an improved survival outcome.

LBA has also been present in the development of novel biopharmaceuticals such as aprosulate and equol. The latter is the major intestinally-derived metabolite of the phytoestrogen daidzein (one of the main isoflavones found abundantly in soybeans), which displays high antioxidant and estrogen activities. Equol has already been revealed to be an agent for treating osteoporosis or preventing breast cancer and vascular diseases, among other pathologies (Oe and Kimura, 2009). Unitika (Japan) and the Osaka Municipal Technical Research Institute (Japan) have developed an equol pharmaceutical composition containing LBA as the main component with enhanced biological properties (Oe and Kimura, 2009; Unitika, 2008). These authors found that the administration of LBA promotes plasma equol production in animal laboratory tests, working as an anti-aging supplement in addition to reducing menopause symptoms (Unitika, 2008).

Likewise, a LBA-based heparin analogue (Aprosulate) has been also devised in recent years as a new amide-derived antithrombotic agent with lower risk of bleeding than unfractionated heparin or low molecular weight heparins (Hoppensteadt et al., 1988; Ofosu et al., 1992; Raake et al., 1989). This sulphated bis-lactobionic acid amide was found to be 25% as effective as unfractionated heparin in its ability to delay both the activation of factor X and the onset of prothrombin activation (Béguin et al., 1991; Ofosu et al., 1992). Klöcking et al. (1991) have additionally shown that the administration of bis-lactobionic acid amides increases the release of the tissue-type plasminogen activator, thereby inducing fibrinolysis. Unfortunately, however, further development of aprosulate as a novel anticoagulant drug was abandoned after Phase II clinical trials, as it was associated with liver toxicity (Coombe et al., 2012; Krylov et al., 2011), despite the reduced coagulation and platelet functions achieved during Phase I clinical trials (Schenk et al. 1999).

Another major application for LBA involves its use as the core compound of preservation solutions employed in organ transplantation protocols which suppress tissue damage caused by free radicals during organ storage (D'Alessandro et al., 1999; Hart et al., 2002). The University of Wisconsin solution (UW solution), also known as ViaSpan (commercial name) or Belzer solution, was developed in the late 1980s by Belzer and colleagues as one of the first intracellular-like preservation media for cold storage of organs before transplantation (Belzer et al., 1992; Southard and Belzer, 1995). This preservation solution has now been adopted worldwide as the gold standard clinical dissolution for organ perfusion in transplantation medicine (Southard, 2002; Upadhya and Strasberg, 2000). UW solution is known to effectively prevent ischemia and reperfusion injury, playing a key role in organ cytoprotection and viability prior to transplantation (Hart et al., 2002). As the central core component of UW solution, LBA works as a cell impermeant agent during the cold storage of organs, as it provides osmotic support and prevents cell swelling (D'Alessandro et al., 1999; Sumimoto and Kamada, 1990). Besides, LBA acts as an antioxidant by scavenging free hydroxyl radicals generated in the medium (Charloux et al., 1995; Hart et al., 2002), as well as a cryptic inhibitor of matrix metalloproteinases due to the fact that it may bind to the divalent zinc atom in the core of these gelatinases and hence inhibit their activity (Upadhya and Strasberg, 2000). These undisputable biological properties offered by LBA have been recently employed for the development of cryoprotectant suspensions containing LBA that are clinically used in cell therapies as compatibles vehicles (Hope et al., 2012).

3.2 Cosmetics field

The cosmetics industry is currently employing LBA as the key active component of novel anti-aging and regenerative skin-care products due to its therapeutic efficacy. The use of LBA in cosmetics has grown three- to five-fold since it was launched commercially, with LBA presenting a major market niche as a novel and essential protective component of skin-care formulations. In fact, the cosmetics company NeoStrata (USA) has devised a broad portfolio of skin-care products based on the unique features offered by LBA in a market which has grown enormously over the past few years, generating US sales of about $1 billion (West, 2004a). As a cosmetic ingredient, LBA offers multiple benefits for the therapeutic treatment of dermatological pathologies such as atopic dermatitis and rosacea (Briden and Green, 2006; Hatano et al., 2009) or can even be employed in anti-acne treatments (Decker and Graber, 2012). Some thirty years ago, Van Scott and Yu found that topical application of alpha-hydroxyacids showed a very specific effect on hyperkeratinization (Green et al., 2009; Yu and Van Scott, 2004). Recently, polyhydroxy acids such as LBA have emerged as the new generation of alpha-hydroxyacids which not only offer similar therapeutic effects, but also reduce side effects like irritation and stinging derived from the use of traditional hydroxy acids, suggesting that polyhydroxy acids may be employed as suitable molecules for the treatment of sensitive skin (Decker and Graber, 2012; Green, 2005). Tasic-Kostov et al. (2010) have clinically evidenced the lack of both skin irritation and skin barrier impairment displayed by LBA compared to classical hydroxyacids such as glycolic acid.

As a versatile substance in cosmetics, LBA is employed as an anti-aging and keratinization agent in cosmeceutical formulations, thus promoting the biosynthesis of glycosaminoglycans or collagen in addition to improving skin thickness and firmness (Green et al., 2008, 2009). Both barrier homeostasis and stratum corneum functionality are clearly improved when LBA is applied topically (Hachem et al., 2010). LBA in fact constitutes an inhibitor of the breakdown of matrix metalloproteinase enzymes due to metal chelation, thus reducing the appearance of photoageing and wrinkles (Grimes et al., 2004). In addition to LBA's antioxidant role, it also exhibits strong moisturizing, exfoliative and humectant properties (Yu and Van Scott, 2004).

3.3 Applications of LBA in the chemical industry

LBA is used in the chemical industry as a sugar-based surfactant or as a cobuilder in biodegradable detergents (Gerling, 1998). Its iron-chelating and emulsifying properties have suggested its potential use for many industrial purposes, including its use as an important starting chemical for producing detergents (Gerling, 1998). Novel

alkylaminoamide (Oskarsson et al., 2007), nonionic (Rôzycka-Roszak et al., 2007), catanionic (Bize et al., 2010; Rico-Lattes et al., 2005), lactobionamide (Lebaupain et al., 2006), gemini (Yoshimura et al., 2005), polysiloxane (Zeng et al., 2012) and aldonamide sugar surfactants (Wilk et al., 2002) have thus been recently developed as novel biodegradable LBA-based surfactants which provide improved surface and performance properties, as well as a reduced environmental impact. In particular, Gerling et al. (1996) have developed LBA amide compositions (formed by reacting LBA with mixtures of primary fatty acids) with good foam stabilizing, cleaning, emulsifying and softening properties in aqueous systems for their application in detergents, cleaning agents and cosmetic formulations. Environmentally-friendly compositions comprising LBA N-alkylamides have likewise been proposed as corrosion prevention agents for their use in many metalworking operations (Gerling et al., 1998). LBA is also being specifically used as an active ingredient in a novel antibacterial agent composition which confers excellent preservation stability on consumer goods as such as foods, raw materials, detergents, cosmetic products and medicines (Araki et al., 2006). In another approach, LBA has been proposed as a building block for the biocatalytic synthesis of novel polymers with possible industrial applications (Kakasi-Zsurka et al., 2011).

Aside from the aforementioned applications, LBA can play a key role as a functionalization agent for the synthesis of innovative industrial systems such as functionalized carbon nanotubes with LBA amide amphiphile molecules which are capable of adsorbing proteins (Feng et al., 2011). Interestingly, LBA-based derivatives could serve as a potential protein cross-linker for both food and non-food applications (van Wijk et al., 2006). Zhou et al. (2006) have reported the direct electrochemistry and electrocatalysis of hemoglobin in a LBA film, which suggests that a new kind of unmediated third-generation hydrogen peroxide biosensor might be developed.

3.4 Food applications

The use of LBA as a food additive has also received growing attention from the food industry in recent years. It can serve as an anti-oxidant, stabilizer or gelling agent in bakery products (Gerling, 1998; Gutiérrez et al., 2012), an acidifier agent in fermented milk products (Faergemand et al., 2012), an aging inhibitor for bread (Oe and Kimura, 2011) and as a mineral absorption enhancer in dairy desserts (Oe and Kimura, 2008). LBA has also been proposed as a technological feed additive for laying hens with the aim of improving eggshell qualities by boosting calcium absorption (Kimura, 2006). Equally importantly, novel non-dairy beverages (Nielsen, 2007), milk-based beverages (Oe et al., 2007) as well as

cheeses (Nielsen and Hoeier, 2009) containing calcium lactobionate have recently been developed to provide a valuable approach for calcium supplementation. Specifically, the incorporation of LBA into foods may stimulate intestinal Ca^{2+} (Brommage et al., 1993) or mineral absorption (Baldwin et al., 2007), thereby exerting a clearly health-promoting influence. Experiments carried out by Oe et al. (2008) have shown that calcium absorption in rats fed a diet supplemented with LBA was higher than in rats fed a control diet, which underlines the potential role of LBA as an agent for preventing and/or treating health disorders associated with calcium deficiency.

The dairy industry has been particularly involved in recent years in the development and implementation of innovative manufacturing processes including LBA as a key ingredient in novel dairy-making technologies (Koka et al., 2005; Merrill and Singh, 2011; Novozymes, 2009). In fact, adding LBA either directly (up to 10% of the mix) or indirectly (generated *in situ* by an added lactose oxidase during the course of the process) enables enhanced production yields, lower processing times and cost savings in manufacturing (Koka et al., 2005). Coagulation may be induced during cheese-making without using culture starters and/or rennet, thereby reducing the need for these costly additional ingredients. Furthermore, the use of LBA as an ingredient in dairy-making and in other product manufacturing processes also provides extra functional properties and sensory attributes through the reduction of undesirable Maillard browning in cooking products (Merrill and Singh, 2011). Above and beyond these applications, a novel LBA-based flavour enhancer for foods or beverages has been also reported by Walter and Begli (2011), which particularly underlines LBA's versatility. Baldwin et al. (2004) have also devised an antioxidant composition containing LBA and siderophores as key elements for retarding lipid oxidation in food products. The role of LBA as a water-holding capacity agent in meat products submitted to thawing and/or cooking processes has recently been reported for the first time, resulting in higher industrial product yields and water contents after treating meat products with LBA (Nielsen, 2009).

In addition to the aforementioned technological role, LBA may also exert potential prebiotic effects as a bioactive ingredient in functional foods, as this organic acid, which is resistant to digestive enzymes, is poorly absorbed in the small intestine and can be subsequently fermented by the gastrointestinal microflora (Saarela et al., 2003; Schaafsma, 2008). In fact, Saarela et al. (2003) have evaluated the potential health benefits of LBA as a prebiotic substance, although unfortunately the enhancement on its functional properties could not be proved. Nonetheless, experiments carried out by Kontula et al. (2000) have confirmed that LBA may promote both the growth and *in vitro* intestinal adhesion of

Lactobacillus cellobiosus (a lactic acid bacteria isolated from the human colonic intestine) compared to other carbohydrate sources, thereby underlining its potential role as a prebiotic substance. Although LBA rarely occurs in nature, it has been isolated in the upper layer from “Caspian Sea yoghurt”, a yoghurt marketed in Japan (Kiryu et al., 2009). These authors have estimated an individual intake of 0.5 to 1.0 g of LBA per year when consuming 100 g of the yoghurt per day, which suggests that the presence of this organic acid in dairy-fermented products could be more common than previously reported (Kiryu et al., 2009).

Although the role of LBA as a food preservative is anticipated, its use as calcium lactobionate (E-399) has already been approved in the USA by the FDA (FDA, 2011), whereas its status in the EU is still pending its approval as a food additive (Playne and Crittenden, 2009; Schaafsma, 2008). Further assessments regarding its long-term safety and human exposure are thus necessary before its approval as a novel food by the European Food Safety Agency (EFSA). Nevertheless, studies carried out by Van Dokkum et al. (1994) have attempted to determine the effect on the human health of exposure to the continued consumption of LBA for one week. These authors concluded that amounts of up to 24 g of LBA per day were well tolerated without producing serious adverse effects on human health. Evidence of LBA fermentation in the human colon was also found, suggesting a likely role of LBA as a prebiotic substance (Schaafsma, 2008; Van Dokkum et al., 1994).

4. Lactobionic acid bio-production methods

4.1 Microbial fermentation strategies for lactobionic acid production.

The use of microbial cell factories for LBA bio-production has become a feasible way to overcome certain drawbacks associated with chemical or enzymatic approaches. Despite the apparent advantages offered, the industrial production of bionic acids by fermentation has barely been explored to date compared to other conventional organic acids. From an industrial point of view, the titer obtained through any biotechnological approach must be at least 50-100 g/L in order to achieve product concentrations comparable to those obtained by means of chemical processes (Pollard and Woodley, 2007).

The production of LBA by bacteria was first discovered in the late 1940s by Stodola and Lockwood (1947) after a wide screening for LBA-producing ability in *Pseudomonas* species. In fact, microbial production and consumption of bionic acids (maltobionic, aldonic and lactobionic) have been explored since the 1950s (Stodola and Jackson, 1950). Research studies by Kluyver et al. (1951) and Stodola and Lockwood (1947) both underlined the importance of *Pseudomonas* species as the main bionic producer microorganisms. Strains such as *P. mucidolens, P. myxogenes* or *P. fluorescens* were found to be able to oxidize lactose

directly without the need for prior hydrolysis or phosphorolysis. *Pseudomonas taetrolens* (formerly known as *P. graveolens*) displayed the highest oxidative ability, with a yield of 75% in shake-flask culture after 165 h (Stodola and Lockwood, 1947). Furthermore, experiments carried out by Sternberg and Lockwood (1969) demonstrated that *P. taetrolens* was able to oxidize not only 1-4 disaccharides (i.e. lactose or maltose), but also isomaltose (α 1-6 disaccharide) to their corresponding bionic acids without hydrolyzing the glycosidic linkage. This latter feature was subsequently explored by Miyake and Sato (1975), who reported the production of aldonic acids from starch sugars after a two-stage process, including a first growth of the *Pseudomonas* species followed by the subsequent enzymatic synthesis of the target compounds by the permeabilized cells. Pseudomonads are therefore the ideal microbial platforms for lactose oxidation, which essentially means the production of LBA, as they lack the genes encoding the β-galactosidase enzyme or lactose transporters.

A slow consumption phenomenon of LBA formed by *Pseudomonas* strains was described at a later stage cultivation (>16 days) under prolonged nutrient starvation conditions (Kluyver et al., 1951). Kluyver et al. (1951) showed that some LBA-producing *Pseudomonas* species such as *P. quercito-pyrogallica*, *P. calco-acetica* and *P. aromatica*, which converted lactose into LBA with yields of 85-90%, were able to consume bionic acids in media in which these compounds were the sole carbon source. However, further studies by Bentley and Slechta (1960) concluded that the rate of bionic formation by *P. quercito-pyrogallica* appeared to greatly exceed that of utilization, a result which was not observed in short-term growth experiments. These authors proposed a general mechanism for bionic acid formation by *Pseudomonas* strains comprising the appearance of a lactone intermediate prior to the formation of the aldonic acid, which was further confirmed by Nishizuka and Hayaishi (1962).

In *Pseudomonas* species, LBA is formed via the lactose oxidation pathway, in which a membrane-bound dehydrogenase system catalyzes this single biotransformation (Fig. 3A). A lactose dehydrogenase enzyme first catalyzes the oxidation of the lactose to a lactone intermediate (lactobiono-δ-lactone) which is subsequently hydrolyzed (its carbonyl group) by a lactonase into LBA (Nishizuka et al., 1960; Nishizuka and Hayaishi, 1962). The lactose dehydrogenase, biochemically characterized by Nishizuka and Hayaishi (1962) from *P. taetrolens* cells, contains flavin adenine dinucleotide as a prosthetic group and is closely associated with a hemoprotein electron transfer system. This flavoprotein, localized in the particulate fraction of *P. taetrolens* cells, does not use oxygen as direct electron acceptor and presents an optimum pH at 5.6, with a K_m value for lactose of 11 mM. Although the purified enzyme may oxidize several aldoses, such as maltose, glucose, mannose, arabinose,

cellobiose, fucose, xylose, ribose and galactose, the specific enzyme activities vary with growth conditions. Conversely, lactonase presents an optimum pH at 6.5-6.7, with a K_m value for lactose of 20 mM (Nishizuka and Hayaishi, 1962). A similar mechanism is responsible for the biosynthesis of LBA by purified fungal enzymes, e.g. the cellobiose dehydrogenase from *Sclerotium rolfsii*, but in turn the intermediate lactobiono-δ-lactone is spontaneously hydrolyzed here to LBA without the involvement of the lactonase enzyme (Ludwig et al, 2004; Van Hecke et al., 2009a).

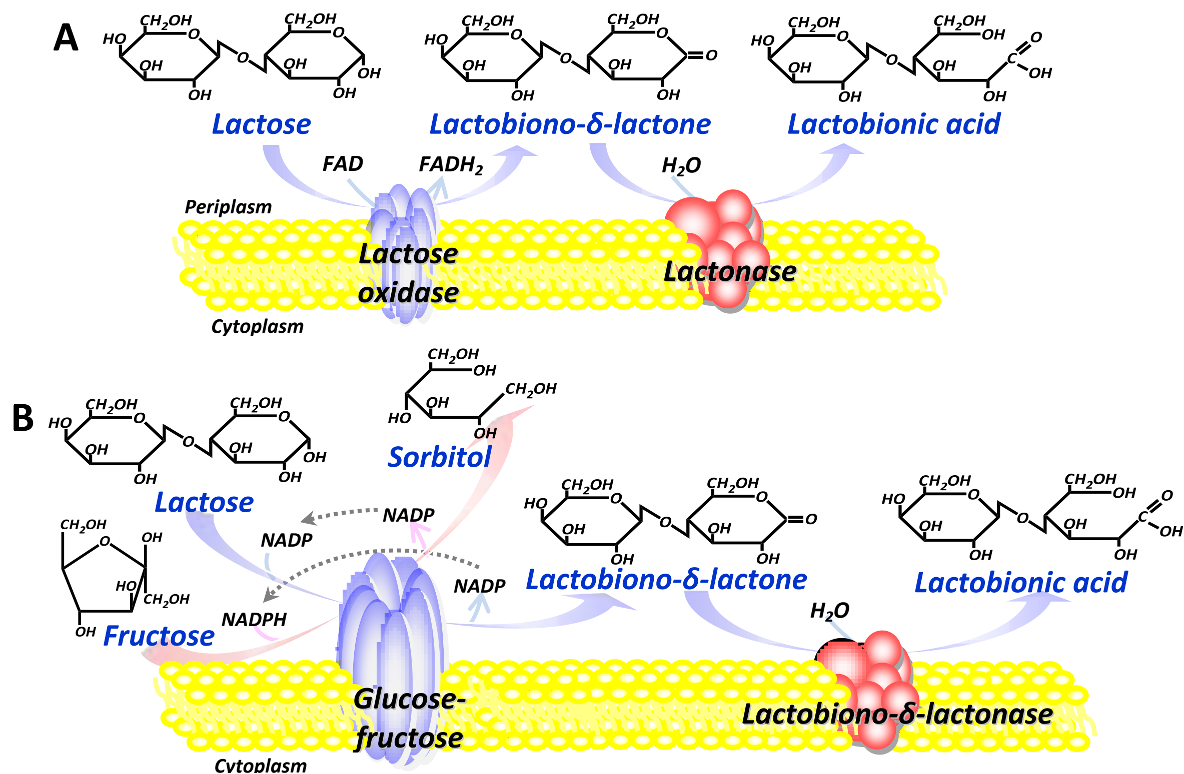


Figure 3. Oxidation pathway for LBA production in bacterial strains. Bioconversion of lactose to lactobionic acid by *Pseudomonas* species is carried out through a membrane-located dehydrogenase system which mediates lactose oxidation and subsequent hydrolysis by lactonase to yield LBA (A). Reaction schemes of a bi-substrate enzymatic system carried out by glucose-fructose oxidoreductase in *Z. mobilis* for the production of LBA and sorbitol (B).

Vakil and Shahani (1969) found that LBA also constitutes an intermediate in the lactose metabolism of *Streptococcus lactis*. LBA is first produced from lactose and is then hydrolyzed by *S. lactis* to gluconic acid and galactose via the β-galactosidase enzyme in order to be further metabolized through both the Embden Meyerhof and hexose-monophosphate shunt pathways (Vakil and Shahani, 1969).

Evidence of the production of LBA by filamentous fungi has also been reported by Bucek et al. (1956). These authors studied the formation of LBA from lactose and its subsequent consumption by *Penicillium chrysogenum* in shake-flask cultures with a LBA yield of only 50% after 120 h, which suggests the presence of residual lactose oxidase activity in this fungus. Their preliminary results also concluded that this organic acid seems to be only an intermediate within the lactose metabolism of this fungus. In addition, the lactose-oxidizing ability has been also found in a red alga, *Iridophycus flaccidum,* which possesses a carbohydrate oxidase with wide substrate specificity. The purified carbohydrate oxidase from this photosynthetic organism was capable of oxidizing glucose, galactose, lactose, maltose and cellobiose at an optimum pH of 5.0 (Bean and Hassid, 1956). Although there is no established consensus regarding the role of sugar oxidation in living cells, the presence of one consumption stage upon oxidation might suggest a key function of this phenomenon in carbon metabolism under nutrient starvation conditions.

To date, most methodologies related to the microbial production of LBA are based on the oxidation of refined lactose from media that is both costly and complex (Table 5). However, LBA can be obtained from an inexpensive feedstock such as cheese whey through a fermentation process carried out by *P. taetrolens* (Alonso et al., 2011, 2012a). The use of cheese whey as an inexpensive source for LBA bio-production undoubtedly suggests a promising strategy to achieve cost-effective whey upgrading. From a practical point of view, this system also provides an environmentally-friendly and competitive alternative to costly and laborious upstream processing steps for LBA production from chemical or enzymatic methodologies based on refined lactose oxidation. In this system, LBA constitutes a non-growth related metabolite formed as a consequence of lactose oxidation from whey by *P. taetrolens* cells (Alonso et al., 2011, 2012a). *P. taetrolens* constitutes a non-pathogenic wild-type microorganism, commonly found in spoiled foods as a natural niche (Levine and Anderson, 1931; Tompkin and Shaparis, 1972; West, 2004b), which belongs taxonomically to the *P. chlororaphis* group (Anzai et al., 2000). The enzymes from this microorganism are even employed industrially for the synthesis of glutamic acid derivatives (Sugiyama et al., 2005). Despite being a wild-type LBA-producing microorganism, efficient LBA production yields, titers and productivity values can be achieved compared to LBA bio-production approaches carried out by other bacteria such as *Burkholderia cepacia* or *Zymomonas mobilis* which were performed under resting cell conditions involving high initial cell densities (Malvessi et al., 2012; Murakami et al., 2002, 2003, 2006b; Pedruzzi et al., 2011) (Table 5). In fact, increasing the inoculum size was shown to improve LBA productivity and hence reduce the onset of the production phase (Alonso et al., 2011). The use of different

bioconversion strategies at the bioreactor scale such as pH-shift or high-cell density conditions may undoubtedly overcome certain drawbacks which could limit LBA productivity by *P. taetrolens* cells (Alonso et al., 2011, 2012b).

The deleterious influence of an over-supply of oxygen on bioconversion performance by *P. taetrolens* has been studied by Alonso et al. (2012a), who concluded that LBA production from whey was negatively influenced by both high aeration and agitation rates. High agitation rates schemes stimulated cell growth, increased pH-shift values and the oxygen uptake rate by *P. taetrolens* cells, whereas LBA production was negatively affected. Mild agitation rates (350 rpm) were found to be the optimal agitation strategy during cultivation, which increased LBA productivity 1.2-fold (0.58-0.70 g/L.h) compared to that achieved at 1000 rpm. Furthermore, aeration rates higher than 1.5 Lpm neither stimulated cell growth nor LBA production (22% lower for an aeration rate of 2 Lpm), the best oxygen supply conditions being found to be 0.5 Lpm. Both the supply and control of dissolved oxygen at the appropriate level have thus been revealed as key bioprocess parameters for enhanced LBA production, representing essential information for large scale implementation (Alonso et al., 2012a).

Fermentation performance for LBA production is also strongly related to the physiological status of the *P. taetrolens* cells used as inocula. Specifically, their central core physiology may suffer from inappropriate bioprocessing conditions such as seed culture age or microenvironmental pH (Alonso et al., 2012b, 2012c). Alonso et al. (2012b) established the best criteria for harvesting cells for enhanced LBA production, besides revealing how cells may respond to environmental stress due to prolonged exposure to extreme acidic conditions, i.e. pH<3.5 (Alonso et al., 2012b). Fermentation performance, which essentially results in a higher LBA titer, may thus be influenced by the physiological heterogeneity of the seed culture, which is attributable to the time point of harvesting in the culture used as inocula for bioreactor cultivations. Seed culture age, which essentially reflects the physiological status displayed by *P. taetrolens* cells, may strongly influence not only overall fermentation performance, but also LBA productivity.

Table 5. Comparison of research studies on the biotechnological production of LBA.

Microorganism	*Culture medium*	*Cultivation strategy and operating mode*	*Inoculum mode*	*Culture time (h)*	*Lactobionic acid titer (g/L)*	*Specific productivity (g/g.h)*	*Productivity (g/L.h)*	*Yield $_{L/LBA}$ (%)*	*Reference*
Pseudomonas sp. LS13-1 [a]	Lactose, salts and peptone	Fed-batch in a 2-L bioreactor	Seed culture (10% v/v)	155	290	-	1.87	90	Miyamoto et al. (2000)
Burkholderia cepacia	Lactose, salts, peptone, yeast extract and glucose	Fed-batch in a 2-L bioreactor	Seed culture (10% v/v)	50	178	-	3.56	85	Meiberg et al. (1990)
Burkholderia cepacia No. 24 [a]	Lactose, salts, peptone and yeast extract	Fed-batch in shake-flask	Resting cells	240	400	-	1.67	100	Murakami et al. (2003)
Burkholderia cepacia No. 24 [a]	Lactose, salts, corn steep liquor and yeast extract	Batch in shake-flask	Resting cells	27	150	-	5.55	~100	Murakami et al. (2006b)
Zymomonas mobilis [b]	Lactose	Batch in a 0.15-L bioreactor	Permeabilized resting free-cells	22	125	0.80	5.80	100	Pedruzzi et al. (2011)
Zymomonas mobilis [b]	Fructose, lactose	Batch in a 0.6-L bioreactor	Permeabilized resting free-cells	24	182	0.3	7.6	78	Malvessi et al. (2012)
Acetobacter orientalis	Lactose, glucose, salts, complex nitrogen sources and yeast extract	Batch in shake-flask	Resting cells	91	49	-	0.54	98	Kiryu et al. (2012b)
Pseudomonas taetrolens	Whey	Batch in a 2-L bioreactor	Seed culture (10% v/v)	58	42	0.56	0.70	100	Alonso et al. (2011)
Pseudomonas taetrolens	Whey	Batch in a 2-L bioreactor	Seed culture (30% v/v)	30	42	0.94	1.27	100	Alonso et al. (2012a)
Pseudomonas taetrolens	Whey	Batch in shake-flask	Resting cells	60	42	0.42	0.70	100	Alonso et al. (2012b)

-: not available; [a] mutant strain; [b] permeabilized cells

The lack of an adaptive response from the *P. taetrolens* cells, comprised in the seed culture, clearly impaired cellular growth, as well as the onset of the production phase (Alonso et al., 2012b). In fact, prolonged-time seed cultures (>12-h) employed as inocula affected microbial adaptation, resulting in the presence of lag-phases along with reduced fermentation efficiencies in terms of LBA productivity, being 1.55-, 2.3- and 3.5-fold (0.45, 0.30 and 0.2 g/L.h for 24-, 36- and 48-h seed cultures, respectively) lower than those obtained using the youngest seed culture (0.70 g/L.h for a 12-h seed culture) (Alonso et al., 2012b). Results also revealed that *P. taetrolens* cells suffered a progressive change in the physiological status of the cells under the uncontrolled-pH conditions encountered at the shake-flask scale (pH=3.35), suggesting that microenvironmental pH constitutes a key bioprocessing factor in LBA microbial production (Alonso et al., 2012b). Production schemes were thus influenced by the operational pH employed, with asynchronous LBA production from damaged and metabolically active subpopulations at pH values lower than 6.0, suggesting a strong influence of the pH-control strategy on the fermentative LBA production (Alonso et al., 2012d). Unlike cultivations performed at 6.5, both pH-shift and pH-stat cultivation strategies performed at pH values lower than 6.0 resulted in decreased LBA yields. Whereas the cellular response showed a stress-induced physiological response under acidic conditions, healthy functional cells were predominant at medium operational pH values (6.5-7.0) (Alonso et al., 2012d). *P. taetrolens* displayed accordingly a robust physiological status at initial pH value of 6.5, resulting in enhanced bioconversion yield as well as LBA productivity.

Inappropriate bioprocessing conditions undoubtedly play a key role in LBA production by *P. taetrolens* cells, seeing as stress-induced physiological responses lead to the collapse of cellular functions. The importance of physiological heterogeneity thus lies in variations in cell capacities which may lead to undesired damaged and dead subpopulations of reduced efficiency during microbial fermentation (Fig. 4). In fact, the biotechnological industry has recognized the importance of detailed physiological knowledge and bioprocess characterization during metabolite production at a population level (Díaz et al., 2010; Lencastre Fernandes et al., 2011).

In this context, the aforementioned approach underlines the suitability of cheese whey as an inexpensive source for LBA production by a wild-type strain (Alonso et al., 2011, 2012b), unlike research studies addressing LBA production on synthetic culture media carried out by mutant strains (Miyamoto et al., 2000; Murakami et al., 2002, 2003, 2006b). In terms of productivity, high-level production of LBA from nutrient-rich media by *Pseudomonas* sp. LS13-1 mutants was achieved under fed-batch fermentation supplemented with 15 g/L of

peptone as the nitrogen source, obtaining a LBA titer of 290 g/L after 155 h, with a volumetric productivity of 1.87 g/L.h (Miyamoto et al., 2000). However, the use of mutant strains on an industrial scale is discouraged due to their genetic instability if a rational method such as metabolic engineering is not employed for their modification (Sauer and Mattanovich, 2012).

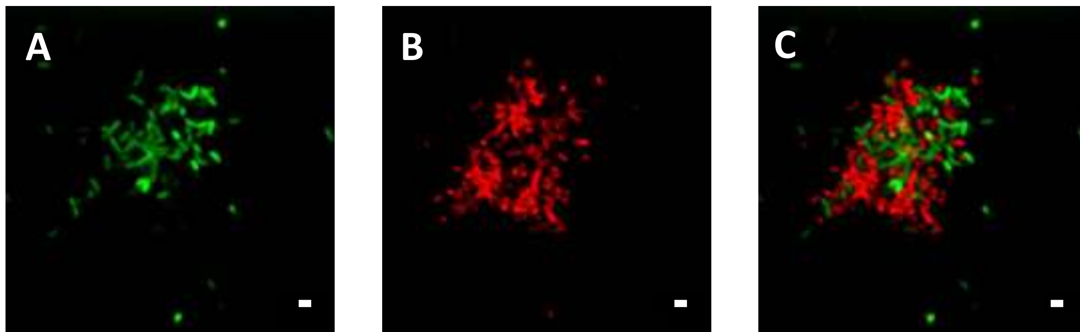


Figure 4. Confocal fluorescence images of *P. taetrolens* cells during fermentative LBA production. The green-fluorescent image represents the signal of metabolically active cells (A), the red-fluorescence image represents dead cells (B), and the overlay of the two previous images (C). Metabolically active cells were stained green (cFDA positive cells, green cells) whereas dead cells (PI positive cells, red cells) were stained red. Scale bars: 2 µm.

Complex media containing lactose, yeast extract and peptone with mineral supplementation for LBA production have been also investigated by Murakami et al. (2002, 2003, 2006b). These authors used a conventional random mutagenesis and screening methods to obtain bionic acid-producing microbial mutants from a soil sample (Murakami et al., 2002). The resulting mutants of *B. cepacia* No. 24 were found to produce up to 400 g/L of LBA on complex culture media over a period of 240 h, with a volumetric productivity of up to 1.67 g/L.h (Murakami et al., 2003). In addition to their lactose-oxidizing activity, aldonic acids have been prepared from galactose, mannose, xylose and arabinose by washed cells of *B. cepacia* No. 24 (Murakami et al., 2006a). A subsequent study revealed that *B. cepacia* No. 24 even exhibited higher lactose-oxidizing activities owing to the more favourable pH conditions, which improved LBA productivity to a value of 5.55 g/L.h in shake-flask cultivation (Murakami et al., 2006b). However, the exact gene cluster modified by random mutagenesis so far remains undeciphered, unlike metabolic engineering approaches that require a detailed knowledge and identification of the target genes directly or indirectly involved in the formation of LBA. The use of complex and expensive media in addition to the lack of genetic characterization may therefore result in a hurdle for upscaling

this system to the industrial sphere. *B. cepacia* cells were also found to produce a high LBA titer (178 g/L) from a high nutrient-rich medium (containing yeast extract, peptone, glucose, salts and lactose) after 50 h, with a volumetric productivity of 3.56 g/L.h and a yield of 85% in 2-L fed-batch fermentation (Meiberg et al., 1990). However, the pathogenicity of this strain discourages any industrial implementation, thus constituting a major shortcoming for this system.

In view of the pathogenicity of *B. cepacia*, Kiryu et al. (2009, 2012b) have reported the biotechnological production of edible LBA by an acetic acid microorganism*, Acetobacter orientalis*, which was isolated from the upper layer of the Japanese "Caspian Sea yoghurt". *A. orientalis* was able to effectively oxidize 2-10% of lactose at a yield of 97 to 99% under resting cell conditions in nutrient-rich media at the shake-flask scale (Kiryu et al., 2012b). However, these authors reported the production of gluconic and acetic acids by *A. orientalis* as undesirable metabolic by-products due to the presence of glucose in the medium. They have further reported how the membrane-bound dehydrogenase contained in *A. orientalis* may oxidize other monosaccharides (such as glucose, allose and xylose among others) as well as disaccharides, although the highest oxidizing activity corresponded to glucose (Kiryu et al., 2012a). In a preliminary assay, Oe et al. (2008) also investigated the oxidization of lactose to LBA by acetic acid bacteria, concluding that *Gluconobacter cerinus* UTBC-427 showed the strongest lactose-oxidizing activity of the screened microorganisms.

In addition to the aforementioned whole-cell bioconversion approaches performed in submerged fermentation systems, different systems involving permeabilized cells have been evaluated in order to achieve high-level production of LBA (Table 5). Permeabilized free-cells of *Zymomonas mobilis* under resting mode have been indeed employed to produce LBA in a bi-substrate system in which the oxidation of lactose to LBA was coupled with the reduction of fructose to sorbitol (Malvessi et al., 2012; Pedruzzi et al., 2011). As Figure 3B shows, two products (LBA and sorbitol) result from the reaction carried out by the glucose-fructose oxidoreductase enzymatic system contained in the permeabilized cells of *Z. mobilis*. Malvessi et al. (2012) reported that the best enzymatic activities of glucose-fructose oxidoreductase were obtained at a pH of around 6.4 and temperatures ranging from 39 to 45ºC. Results from Pedruzzi et al. (2011) concluded that higher initial concentrations of lactose led to increased activity of this enzymatic system. However, a remaining practical drawback of this enzymatic system for LBA production is the low affinity of the glucose-fructose oxidoreductase enzyme for both lactose and fructose, requiring high levels of substrate concentration to promote high productivities (Pedruzzi et al., 2011). Comparatively, high LBA titers (125-182 g/L) and yields (78-100%) were achieved by using

permeabilized cells of *Z. mobilis* (Malvessi et al., 2012; Pedruzzi et al., 2011), although the amount of LBA produced per g cell mass is worth noting (Table 5). In this regard, these systems displayed impressively high volumetric productivity values in terms of LBA production rates (5.8-7.6 g/L.h), although their specific values (0.3-0.8 g/g.h) were significantly lower than those achieved by *P. taetrolens* cells (>0.94 g/g.h).

Bioconversion of fructose and lactose into sorbitol and LBA with immobilized cells of *Z. mobilis* in calcium-alginate has been also approached (Malvessi et al., 2012; Pedruzzi et al., 2011). Results from Pedruzzi et al. (2011) revealed that there were no marked mass transfer limitations on the rate of LBA formation for calcium-alginate beads with an average diameter of 1.2 mm below a productivity value of 14.9 g/L.h. These authors suggested that additional studies should be performed to verify the long-term mechanical stability of calcium-alginate beads (Pedruzzi et al., 2011). Similar results were obtained by Malvessi et al. (2012), who showed that LBA titers, bioconversion yields and specific productivities were similar to those obtained with permeabilized free-cells. The highest glucose-fructose oxidoreductase activities were found at optimum pH values of 7.0-8.0 and temperatures of 47-50ºC (Malvessi et al., 2012).

As regards economic feasibility, the production of organic acids by microbial fermentation requires productivities greater than 1 g/L.h and final product concentrations above 50 g/L (Yang et al., 2007). Although most of the approaches listed in Table 5 meet these criteria, many of them could not be implemented on a commercial scale due to the fact that their high-level production values were achieved under resting cell conditions, which are not usually employed in the fermentation industry.

4.2 Biocatalytic approaches: enzymatic synthesis of lactobionic acid.

Considerable research efforts have focused in recent years on the enzymatic production of LBA (Table 6 summarizes these enzymatic approaches). In these enzymatic systems, refined lactose is oxidized by specific biocatalytic cascades employing enzymes such as oxidoreductoses (Nordkvist et al., 2007; Satory et al., 1997; Van Hecke et al., 2009). However, these systems are costly processes, as they usually rely on the redox mediator or cofactor regeneration, additionally associated with complex operational upstream steps (Ludwig et al., 2004; Maischberger et al., 2008; Splechtna et al., 2001; Van Hecke et al., 2009). Both enzyme production and further purification stages prior to the biocatalysis process clearly constitute additional drawbacks seeing as these upstream steps are costly, time-consuming and laborious. Figure 5B provides an overview of the different steps involved in these biocatalytic approaches, from the cultivation of the source of the enzyme

to the purification of LBA. Comparatively, enzymatic catalysis usually displays higher LBA titers, production yields and volumetric productivities (Table 5) than those obtained under microbial fermentation (Table 6). Despite the high LBA productivity achieved, enzymatic approaches also feature a lack of selectivity and long-term stability in complex media. Another limitation is the loss in enzymatic activity throughout the biocatalysis process which may impair not only LBA synthesis at later reaction times, but also its industrial implementation.

As Table 6 shows, several approaches for LBA enzymatic synthesis involving different carbohydrate oxidases have been reported to date. Dhariwal et al. (2006) studied the production of LBA by cellobiose dehydrogenase (purified from *S. rolfsii*) in an integrated electrochemical regeneration process using electricity instead oxygen as the final electron acceptor, removing the need for a second enzyme (laccase) in the regeneration system. This biocatalytic system achieved a LBA volumetric productivity of 1.8 g/L.h in a 25-mL electrolysis cell using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) as the redox mediator. However, the lack of information on commercial electrolysis flow cells as well as the low LBA productivity value obtained in comparison to other biocatalytic approaches constitute continuing practical drawbacks for the successful industrial implementation of this promising biocatalytic system (Table 6).

Cellobiose dehydrogenase is an extracellular hemoflavoenzyme produced by several wood-degrading fungi such as *S. rolfsii* (Baminger et al., 2001b) which utilizes oxygen as the terminal electron acceptor, thus leading to the formation of hydrogen peroxide, a reaction by-product that is detrimental to enzyme activity. Nevertheless, this latter drawback can be overcome by the addition of redox mediators (electron acceptors) such as ABTS or DCIP (2,6-dichloroindophenol), which are able to accept electrons from cellobiose dehydrogenase. Likewise, the combination of a regenerating enzyme such as laccase enables the re-oxidation of these redox mediators in a bi-enzymatic cascade (Fig. 6). Baminger et al. (2001a) thus achieved the production of LBA from refined lactose by cellobiose dehydrogenase in a biocatalytic approach in which the presence of an electron acceptor (ABTS) was combined with a regenerating enzyme such as laccase (purified from *Trametes pubescens*). In this biotransformation system, complete lactose bioconversion (100%) and an impressive LBA volumetric productivity of 18 g/L.h were achieved in a batch reactor (50-mL) using DCIP as the redox mediator (Baminger et al., 2001a).

Table 6. Summary of research studies on the enzymatic production of LBA.

Enzymes [a]	*Substrate*	*Redox mediator*	*Bioconversion strategy and operating mode*	*Reaction time (h)*	*Lactobionic titer (g/L)*	*Productivity (g/L.h)*	*Yield $_{L/LBA}$ (%)*	*Reference*
Carbohydrate oxidase (*M. nivale*) + catalase[c]	Whey permeate	n.u.	Pilot-scale batch reactor (600-L)	12	49	4	98	Hua et al. (2007)
Carbohydrate oxidase (*M. nivale*) + catalase[c]	Whey permeate	n.u.	-	5	-	-	90	Budtz et al. (2007)
Carbohydrate oxidase (*M. nivale*) + catalase[c]	Lactose	n.u.	Batch reactor (1-L)	10	49	4.9	98	Nordkvist et al. (2007)
Cellobiose dehydrogenase (*S. rolfsii*) + laccase (*T. pubescens*)	Lactose	DCIP	Batch reactor (3-L)	10	72	7	100	Ludwig et al. (2004)
Cellobiose dehydrogenase (*S. rolfsii*) + laccase (*T. versicolor*)	Lactose	DCIP	Batch reactor (50-mL)	~4	72	18	100	Baminger et al. (2001a)
Cellobiose dehydrogenase (*Trametes versicolor*) + laccase (*T. versicolor*)	Lactose	ABTS	Integrated process: electrochemical enzyme regeneration in an electrolysis cell (25-mL)	-	-	1.8	-	Dhariwal et al. (2006)
Cellobiose dehydrogenase (*S. rolfsii*) + laccase (*T. pubescens*)	Lactose, monosaccharides, GOS	DCIP	Batch reactor (0.1-L)	2.5	40	16	96	Splechtna et al. (2001)
Cellobiose dehydrogenase (*S. rolfsii*) + laccase (*T. pubescens*)	Lactose, monosaccharides, GOS	DCIP	Batch reactor (6-L)	9	55	6	100	Maischberger et al. (2008)

Table 6. (Continued).

Enzymes [a]	*Substrate*	*Redox mediator*	*Bioconversion strategy and operating mode*	*Reaction time (h)*	*Lactobionic titer (g/L)*	*Productivity (g/L.h)*	*Yield $_{L/LBA}$ (%)*	*Reference*
Glucose-fructose oxidoreductase (*Z. mobilis*) [b]	Lactose	n.u.	Batch reactor (20-mL)	60	157	2.6	90	Satory et al. (1997)
Cellobiose dehydrogenase (*S. rolfsii*) + laccase (*T. pubescens*)	Lactose	ABTS	Batch membrane reactor (0.2-L) with bubble-free oxygenation	6	18	3	100	Van Hecke et al. (2009a)
Cellobiose dehydrogenase (*Myriococcum thermophilum*) + laccase (*T. pubescens*)	Lactose	ABTS	Batch membrane reactor (0.2 L) with bubble-free oxygenation	7	18	2.6	100	Van Hecke et al. (2009b)
Cellobiose dehydrogenase (*S. rolfsii*) + laccase (*T. pubescens*)	Lactose	ABTS	Discontinuous membrane reactor (20-L)	12	37	3.1	97	Van Hecke et al. (2011)
Lactose-oxidizing enzyme (*Paraconiothyrium* sp. KD-3)	Lactose	n.u.	Batch reactor (2-mL)	7	100	14	100	Kiryu et al. (2008)
Lactose-oxidizing enzyme (*Paraconiothyrium* sp. KD-3) + catalase (*Aspergillus niger*)	Lactose	n.u.	Batch reactor (2-L)	10-20	100-150	9-11	100	Murakami et al. (2008)

-: not available; n.u.: not used; LBA: lactobionic acid; L: lactose; DCIP: 2,6-dichloroindophenol; ABTS: 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); GOS: galacto-oligosaccharides.

[a] The source of the enzyme is shown in parenthesis.

[b] Dithiothreitol and bovine serum albumin were additionally used as stabilizers.

[c] Commercial catalase enzyme: Catazyme® from Novozymes A/S.

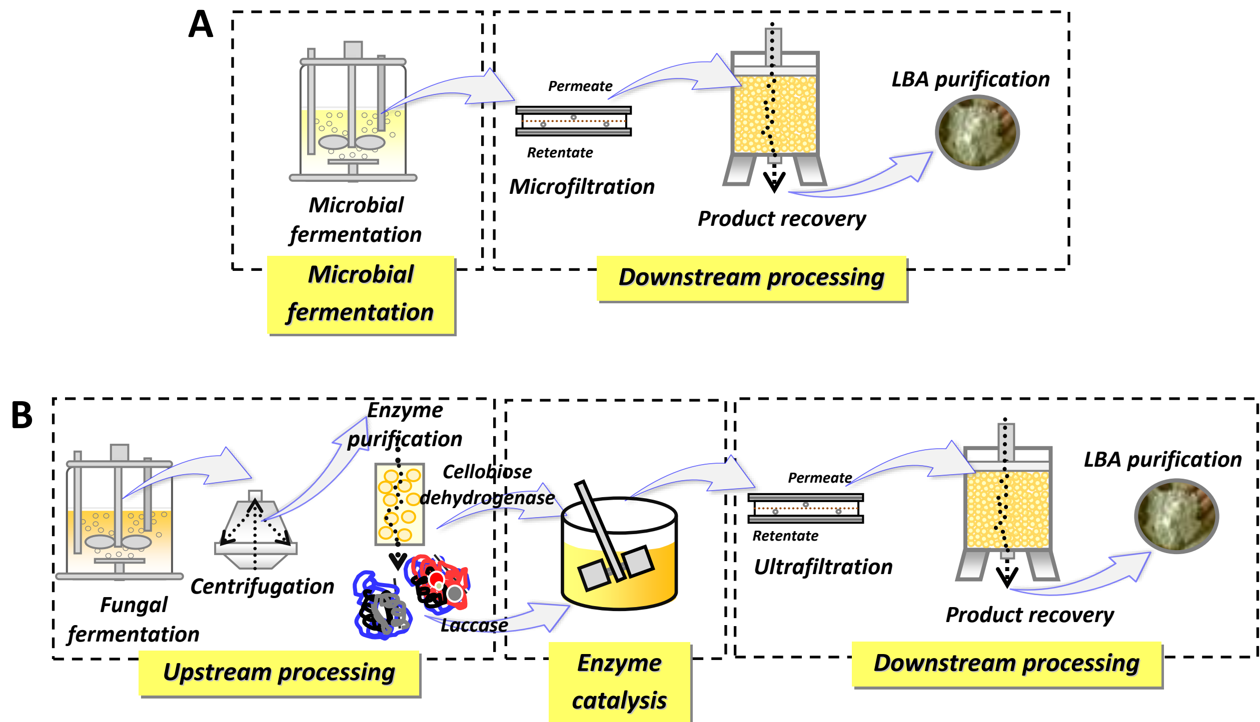


Figure 5. Schematic diagrams illustrating processes for the production of LBA via microbial production (A) and enzymatic synthesis (B).

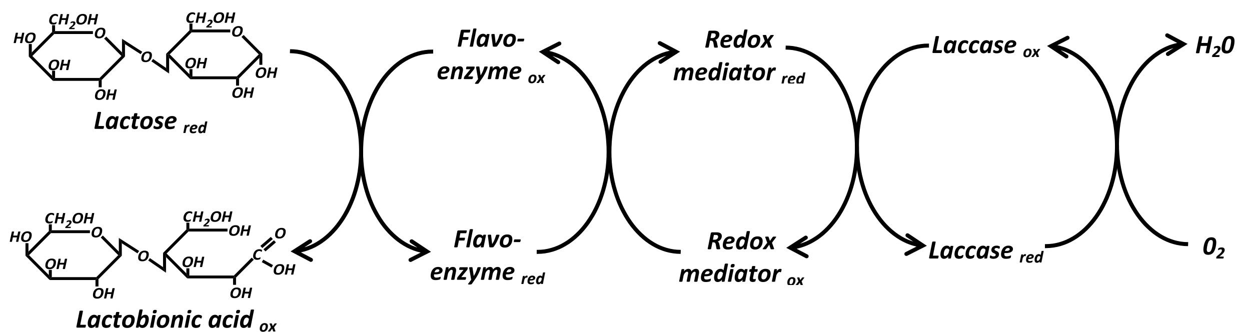


Figure 6. Continuous laccase-catalyzed regeneration of redox mediators that are employed in the oxidation of lactose by a flavo-enzyme (i.e. cellobiose dehydrogenase).

Another approach involving LBA production by cellobiose dehydrogenase was studied by Ludwig et al. (2004), who reported the inactivation of the enzymatic system under increasing dissolved oxygen tension conditions, suggesting a possible deleterious combination of aeration and shearing forces on the stability of cellobiose dehydrogenase and laccase. Another drawback that emerged was the need to remove the redox mediators from the system after biocatalysis by means of adsorption due to their toxicity (Ludwig et al., 2004), a feature commonly found in most cell-free biocatalytic processes using purified

hemoflavoenzymes (Table 6). In terms of volumetric productivity, this biotransformation system, featuring the continuous laccase-catalyzed regeneration of the electron acceptors, displayed a LBA volumetric productivity of 7 g/L.h with complete lactose bioconversion (100%) after 10 h (Ludwig et al., 2004). The inactivation of this bi-enzymatic system under high aeration conditions was overcome using a bubble-free oxygenation strategy (Van Hecke et al, 2009a, 2009b, 2011). These authors studied the discontinuous production of LBA by cellobiose dehydrogenase in a 20-L membrane reactor, obtaining a volumetric productivity of 3.1 g/L.h with a yield of 97% (Van Hecke et al., 2011). Moreover, the modeling and further experimental corroboration of this biotransformation approach under continuous conversion resulted in good enzyme stability (~80% of the initial activity) for 3 days, although long-term operation (>3 days) under non-sterile conditions was unsuccessful (Van Hecke et al., 2011).

Residual lactose from a transgalactosylation process for galacto-oligosaccharide production has also been submitted to a biotransformation process to yield LBA by cellobiose dehydrogenase in an enzymatic regeneration method with productivities ranging from 6 to 16 g/L.h, achieving 96 to 100% lactose bioconversion (Table 6) (Maischberger et al., 2008; Splechtna et al., 2001).

The glucose-fructose oxidoreductase enzyme from *Z. mobilis* has been employed as a biocatalyst for LBA synthesis under a continuous system (Satory et al., 1997). However, the use of a glucose-fructose oxidoreductase to oxidize lactose and fructose results into two products, LBA and sorbitol, respectively. Another disadvantage of this bi-substrate system is its low affinity for lactose, as the K_m value of the enzyme for lactose is 1.2 M, which is 80-fold higher than that corresponding to its physiological substrate (glucose). Owing to the presence of reaction stabilizers, the glucose-fructose oxidoreductase was able to retain its biocatalytic activity for more than 150 h. This latter feature was used for the continuous production of LBA in an ultrafiltration membrane reactor with a productivity of 4.6 g/L.h., which was 1.8-fold higher than that obtained under batch enzymatic conditions (2.6 g/L.h) (Satory et al., 1997).

Nordkvist et al. (2007) studied the production of LBA from lactose by a carbohydrate oxidase which was cloned from *Microdochium nivale* and expressed in *Fusarium venenatum*. This carbohydrate oxidase is a flavoprotein which is able to oxidize several carbohydrates and transfer the electrons to molecular oxygen, thus producing hydrogen peroxide that is subsequently removed by an added catalase (Budtz et al., 2007; Hua et al., 2007; Nordkvist et al., 2007). The addition of commercial catalase clearly constitutes a drawback for this biocatalytic approach despite not requiring any redox mediator. A LBA volumetric

productivity 4.9 g/L.h with a yield of 98% was thus achieved in a batch enzymatic reaction performed at 38ºC under 0.2 Lpm continuous gas (mixture of N_2/O_2) sparging (Nordkvist et al., 2007). However, this biocatalytic system featured a decreasing production rate after 6 h due to enzyme deactivation resulting from the slow addition of 2 M NaOH aimed at maintaining an operational pH of 6.4 (Nordkvist et al., 2007; Villadsen, 2007). The additional non-ideal mixing found by Nordkvist et al. (2007) in a 1-L stirred tank bioreactor was overcome by Hua et al. (2007) employing a pilot-scale batch reactor (600-L) equipped with a rotary jet head system which was able to provide the necessary mixing and mass transfer conditions (Villadsen, 2007). This pilot-scale system yielded a LBA titer of up to 49 g/L from whey permeate in 12 h, demonstrating the scalable potential of this biocatalytic process (Hua et al., 2007).

Murakami et al. (2008) obtained complete lactose conversion (100%) employing a purified extracellular lactose-oxidizing enzyme from *Paraconiothyrium* sp. KD-3 at 40ºC, in which 100-150 g/L of lactose were completely converted to LBA within 10-20 h at a rate of 9-11 g/L.h (Murakami et al., 2008). Sugars such as arabinose, glucose, xylose, galactose and xylobiose as well as cellooligosaccharides may also be oxidized by this lactose-oxidizing enzyme which is a carbohydrate:acceptor oxidoreductase in terms of its biocatalytic specificity (Kiryu et al., 2008). LBA production has been also approached employing an immobilized purified lactose-oxidizing enzyme from *Paraconiothyrium* sp. KD-3 on a cation exchange resin, which was able to oxidize up to 185 g/L of lactose (Nakano et al., 2010). However the continuous repeated batch processing of the immobilized enzyme resulted in a decrease in biocatalytic activity due to enzymatic inactivation by the hydrogen peroxide produced during the reaction (Nakano et al., 2010).

In an industrial context, an enzymatic mixture called LactoYIELD was launched onto the market in 2009 as a result of the strategic alliance achieved between the Danish companies Chr. Hansen and Novozymes A/S in 2002 (Novozymes, 2009). This standardized enzymatic mixture allows cheese manufacturers to convert lactose from cheese whey into LBA. The resulting reaction product, calcium lactobionate, can thus be used as an ingredient in the formulation of dairy products such as cheeses or desserts in order to increase adhesive gelling properties, reduce water loss and replace skim milk powder. Furthermore, it can become an interesting source for calcium supplementation of dairy products, providing higher solubility and stability properties without the off-taste resulting from other calcium sources (Novozymes, 2009).

5. Downstream processing of lactobionic acid.

The elucidation of a downstream processing methodology after the bioconversion process that is both suitable and feasible could guarantee the successful implementation of LBA bio-production on an industrial scale. Considering that the recovery process depends primarily on the nature of the matrix employed for LBA production, media components and metabolites from the bioconversion broth could constitute a drawback when purifying the desired target compared to the lesser effort required for downstream processing of LBA produced after catalytic approaches based on refined lactose.

The past few years have seen the development of new approaches for the downstream processing of LBA; Table 7 summarizes different downstream processing approaches for LBA purification. In particular, several attempts have been made to separate and purify LBA after bioconversion processes. Subsequent to cell harvesting, recovery of LBA from the bioconversion broth could thus be accomplished by conventional methods involving precipitation, extraction or adsorption using ion-exchange resins (Pedruzzi et al., 2008). Among the purification methods that have been explored, ion-exchange separation of LBA has been chosen as the preferred downstream processing approach (Table 7). Higher recovery yields were thus achieved under LBA titers of 40-50 g/L through ion-exchange, whereas titers above 100 g/L might need innovative downstream processing approaches such as simulated moving bed technology (Table 7).

Table 7. Summary of downstream approaches employed for the purification and recovery of LBA.

Approach	*Matrix*	*Source*	*LBA concentration (g/L)*	*Recovery (%)*	*Reference*
Crystallization + ion exchange chromatography	Synthetic solution	Synthetic	200	79	Jones and Ho (2002)
Ion exchange chromatography	Synthetic solution	Synthetic	50	100	Pedruzzi et al. (2008)
Ion exchange chromatography	Reaction solution	Enzymatic synthesis	40	100	Splechtna et al. (2001)
Simulated moving bed technology	Bioconversion broth	Bioconversion using permeabilized cells of *Z. mobilis*	125	100	Borges da Silva et al. (2011)
Electrodialysis	Bioconversion broth	Bioconversion using permeabilized cells of *Z. mobilis*	20	38.7	Peretti et al. (2009)

The first attempt to purify LBA after microbial fermentation was a reactive extraction method proposed by Lockwood and Stodola (1950) through precipitation with ethanol.

Murakami et al. (2003) also added ethanol to the culture supernatant after fermentation, resulting in 98% LBA recovery as precipitate. Crystallization via the addition of ethanol has also been proposed as a purification step after LBA production by Armarego and Chai (2009). Magariello (1956) reported the purification of LBA from an electrolytic reaction system through a first evaporation process to obtain a thick syrup which was then dehydrated by distillation with dioxane and toluene, finally obtaining a dry residue that was further crystallized. Alternatively, the process proposed by Magariello (1956) comprised passing the crude electrolytic reaction solution through acidic cation- and weakly basic anion-exchange resins, recovering the aqueous LBA solution effluent and spray-drying it.

Jones and Ho (2002) proposed an optimized method for LBA separation combining a prior evaporation stage (70ºC for 45 min), a crystallization step through a precipitation method and a final ion-exchange process. The resultant precipitate in the precipitation stage was passed through one or more ion-exchange resins before a final freeze-drying process, resulting in a recovery yield of 79% (Table 7).

The selection of resins for ion-exchange separation of LBA (Pedruzzi et al., 2008) and the use of simulated moving bed technology have also been assessed (Borges da Silva et al., 2011). Specifically, Pedruzzi et al. (2007, 2008) studied different chromatographic systems to perform the separation of quaternary mixture from an enzymatic process carried out by the glucose-fructose oxidoreductase enzyme system. These authors selected a suitable stationary phase to perform the separation of the quaternary mixture (lactose, fructose, sorbitol and LBA) and established the valid parameters to predict not only the quaternary separation by ion resins using water as the eluent, but also the dynamic behaviour of fixed and moving adsorption beds (Pedruzzi et al., 2008). The separation of LBA from a glucose-fructose oxidoreductase enzyme reaction through the use of electrodialysis technique was additionally carried out by Peretti et al. (2009). However, the low yield obtained (38.7%) may relegate this methodology as a LBA downstream processing methodology.

LBA recovery after production processes will undoubtedly be the subject of intensive future research. Developing innovative solutions for cost-effective manufacturing of LBA obviously involves integrated fermentation-separation processes. In general, integrated fermentation-separation processes may improve system productivities as well as producing a relatively pure product stream, thus further simplifying downstream processing (Pollard and Woodley, 2007). In Situ Product Recovery (ISPR) techniques, already implemented for numerous organic acids, may likewise constitute a promising strategy for LBA separation.

6. Biotechnological role and future prospects

In this review, several of the challenges and current commercial applications of LBA have been discussed along with the potential perspectives regarding its particular role as an emerging high value-added organic acid. So far, although LBA is not yet biotechnologically produced on an industrial level, the bio-production of LBA from an inexpensive feedstock such as cheese whey may suggest not only a sustainable, but also a cost-effective alternative for obtaining this high value-added compound. Likewise, the disposal and treatment of cheese whey continues to be a crucial challenge for the dairy industry, not only from the environmental, but also from the economic point of view due to the growing costs of disposal and waste treatment scenarios. Moreover, microbial production of LBA may overcome the major drawbacks associated with chemical and enzymatic approaches such as complex and time-consuming upstream steps. In the foreseeable future, biotechnological production of LBA can be expected to make the transition to the industrial sphere. However, metabolic engineering may constitute a key future issue to overcome rate-limiting steps for the microbial production of LBA, introducing new functions into a host strain or improving LBA production by wild-type strains through enzyme overexpression. In addition, more straightforward downstream processes could also contribute to bioprocess development to meet the foreseeable demand, as well as to reduce the costs associated with bio-based production.

As to LBA applications, both functional foods and nutraceuticals constitute interesting areas in which LBA may undoubtedly play a fundamental role over the coming years, given that, as already mentioned, this polyhydroxy bionic acid provides unique health-promoting functions. However, new data is needed to complete its comprehensive risk assessment before drafting the opinion of panel members on food additives prior to the final adoption by the EFSA of its standby status as a food additive in Europe.

Aside from its role in the food sector, LBA has a great potential as a useful compound in the treatment of hepatic diseases. LBA may thus lead the way to novel therapies based on hepatic targeting of ASGPR, seeing as the potential applications of LBA as a platform for biomaterials or scaffolds in tissue engineering (Peng et al., 2007) or as bio-functionalization agent of nanoparticles used for the treatment of recalcitrant diseases such as liver cancer are really promising (Jain and Jain, 2010; Mi et al. 2007). Furthermore, bio-functionalization of drug delivery systems through LBA affords a promising way to provide sustained release of drugs targeting liver cancer cells (Duan et al., 2011; Huang et al., 2011; Varshosaz et al., 2012). LBA will undoubtedly play a major role in these novel therapies as the core molecule, expanding its usefulness beyond its technological role as an additive in food processing.

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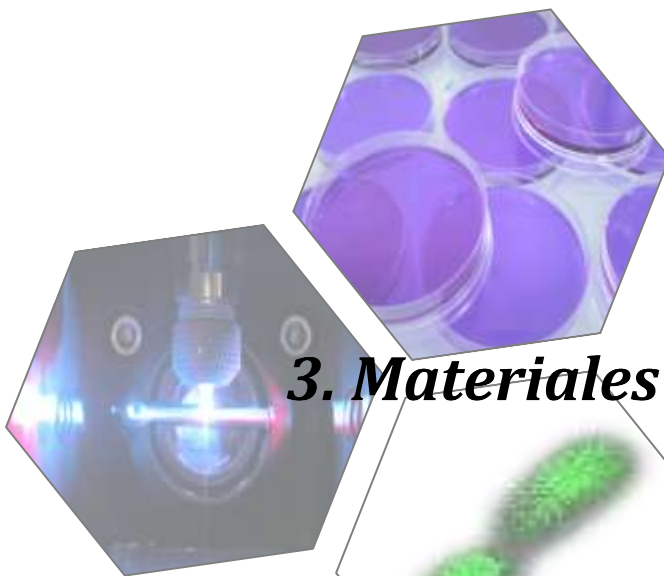


3. Materiales y métodos

3. MATERIALES Y MÉTODOS

3.1. Producción biotecnológica de ácido láctico

3.1.1. Microorganismo de cultivo

Lactobacillus casei ATCC 393, obtenida a partir de la Colección Americana de Cultivos Tipo (Virginia, Estados Unidos) fue mantenida congelada (en solución con 40 % v/v de glicerol a -20ºC) y a 4ºC en placas de Man Rogosa and Sharpe agar (MRS, Biokar Diagnostics, Francia). La cepa fue cultivada en caldo MRS o en placas de agar (con un 2 % p/p de agar).

3.1.2. Preparación del lactosuero de yogurt

El lactosuero residual de yogurt fue obtenido a partir de yogures fuera de la fecha de caducidad, los cuales fueron mezclados previamente con el fin de obtener el lactosuero mediante coagulación térmica, eliminando de esta forma la mayoría de la grasa y las proteínas. A continuación, el resto de lípidos y proteínas fueron retirados de esta fracción líquida mediante centrifugación a 12.000 x *g* (Kubota, modelo 6500, Tokyo, Japón) durante 10 minutos a 4ºC. Posteriormente, el lactosuero se diluyó con agua destilada (en un ratio 1:1 v/v) y se ajustó el pH a 6,5 con NaOH 1 N. Una vez diluido, el lactosuero de yogurt fue esterilizado a través de una microfiltración tangencial con un cassette de membranas de PVDF (Millipore, Massachusetts, Estados Unidos) con un tamaño de poro de 0,22 μm. La Tabla 3.1 recoge la comparación entre los valores físico-químicos determinados experimentalmente de la fracción líquida de partida y del lactosuero utilizado en los experimentos.

Tabla 3.1. Características físico-químicas del lactosuero de yogurt utilizado en las fermentaciones.

	NTK (mg/L)	*[Proteína] (g/L)*	*Densidad (g/L)*	*ºBrix*	*Humedad (%)*	*DQO (mg/L)*	*pH*
Lactosuero de yogurt inicial	580	3.6	1042	13	91.6	90	4.2
Lactosuero de yogurt utilizado en las fermentaciones	309	1.9	1018	6.5	96.1	65	6.5

3.1.3. Condiciones de cultivo

Tras la reactivación de *L. casei* en MRS bajo condiciones microaerófilas, un 10 % (v/v) de este cultivo (en fase de crecimiento exponencial) fue empleado para inocular tanto 90 mL de lactosuero de yogurt como lactosuero de yogurt suplementado con 2,5 g/L de extracto de levadura, ambos cultivos utilizados como preinóculos. Estos preinóculos fueron incubados a 37ºC durante 20 h a 100 rpm en un incubador orbital (Infors HT, modelo Flyer Aerotron, Bottmingen, Suiza). A continuación, estos precultivos fueron empleados para inocular las fermentaciones de lactosuero de yogurt. Las fermentaciones sin control de pH fueron llevadas a cabo en un agitador orbital (100 rpm) con un volumen final de trabajo de 1 L a 37ºC. Mientras que las fermentaciones con control de pH se llevaron a cabo en un biorreactor de 2 L (Bioflo 110, New Brunswick Scientific Co. Inc., Nueva Jersey, Estados Unidos) con un volumen de trabajo de 1 L, equipado con un control digital de la unidad, siendo la temperatura controlada a 37ºC y el pH fue fijado en 6,5 usando NaOH 3 N. La agitación fue fijada en 50 rpm con el fin de mantener el medio de fermentación mezclado, y así evitar la sedimentación tanto de *L. casei* como del ácido láctico formado. Las muestras fueron tomadas a intervalos regulares de 2 h con el fin de determinar la densidad óptica y el pH, mientras que los sobrenadantes libres de células (filtrados por 0,45 µm) fueron almacenados bajo congelación hasta posterior análisis. Las fermentaciones se llevaron a cabo en duplicado como experimentos independientes.

3.1.4. Métodos analíticos

Con objeto de determinar la biomasa celular, las muestras tomadas a lo largo de la evolución de los cultivos fueron centrifugadas durante 5 minutos a 16.000 x *g* (Eppendorf, modelo 5415 D, Hamburgo, Alemania), siendo resuspendido el correspondiente pellet resultante en una solución de NaCl 0,7% (p/v). El crecimiento celular fue medido espectrofotométricamente como densidad óptica a una longitud de onda de 660 nm (Shimazdu, modelo UV 1203). La densidad óptica fue convertida en medidas de biomasa (peso seco) usando la correspondiente curva de calibración previamente determinada, expresando los resultados en gramos por litro (g/L).

El contenido proteico de los sobrenadantes fue determinado de acuerdo con el procedimiento descrito por Bradford (Bradford, 1976) (reactivo Sigma-Aldrich, Steinheim, Alemania) utilizando sero-albúmina bovina como patrón.

Mientras que la lactosa, glucosa, sacarosa y el ácido láctico, presentes en los sobrenadantes tomados a lo largo de la evolución de los cultivos, fueron analizados mediante Cromatografía de Líquidos de Alta Eficacia (HPLC) siguiendo el método descrito por Pedruzzi et al. (2007) con pequeñas modificaciones. En síntesis, se han utilizado las siguientes condiciones analíticas: 0,450 mM H_2SO_4 como fase móvil (pH 3,1), una temperatura de la columna fijada en 75ºC y un flujo de 0,3 mL/min. En la determinación y cuantificación analítica se ha empleado una columna ICSep ICE-ION-300 (Transgenomic Inc., San Jose, California, Estados Unidos), utilizando el índice de refracción como detector en un cromatógrafo Agilent (modelo serie 1200, California, Estados Unidos) (Figura 3.1). Las muestras fueron cuantificadas empleando *standards* externos de grado HPLC obtenidos de las siguientes casas comerciales: lactosa, sacarosa y ácido láctico fueron obtenidos de Chem Service (West Chester, Pennsylvania, Estados Unidos), glucosa de Merck (Darmstadt, Alemania) y etanol de Panreac (Barcelona, España). La adquisición y análisis de datos fue realizada con el software Agilent ChemStation.

Figura 3.1. Cromatógrafo de líquidos de alta eficacia (HPLC) empleado en las determinaciones analíticas.

3.1.5. Monitorización mediante citometría de flujo

La monitorización del estado fisiológico de *L. casei*, se ha efectuado a lo largo de dos procesos fermentativos, con y sin control de pH. Un resumen de los pasos llevados a cabo durante el proceso se muestra en la Figura 3.2. Diferentes muestras fueron tomadas con el fin de determinar el estado fisiológico mediante citometría de flujo y densidad óptica a lo largo de la evolución de ambos procesos fermentativos. Los sobrenadantes fueron almacenados (a -20ºC) hasta el correspondiente análisis de los mismos.

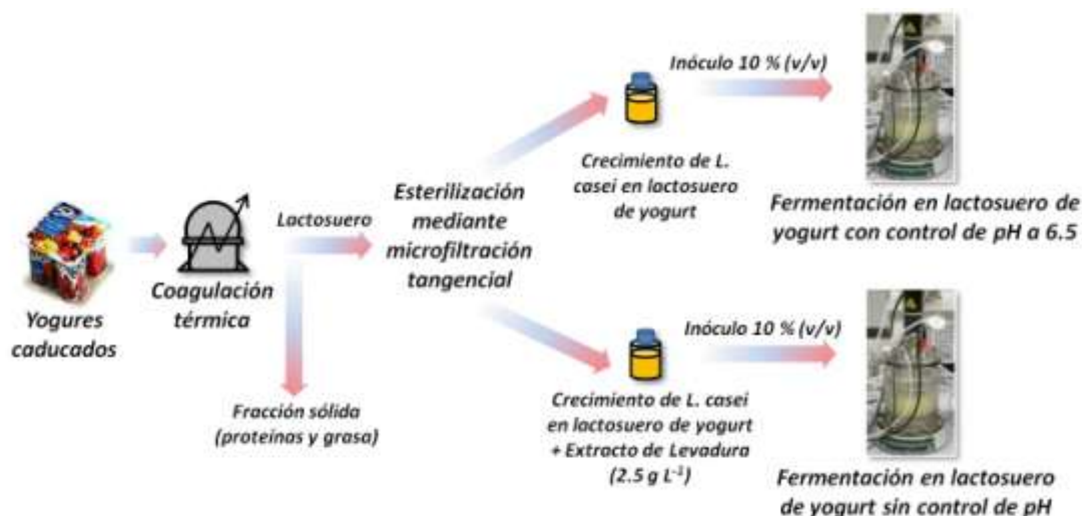


Figura 3.2. Diagrama que esquematiza las etapas previas y fermentaciones llevadas a cabo en el seguimiento y monitorización mediante citometría de flujo. Los precultivos en lactosuero de yogur con y sin extracto de levadura fueron utilizados como inóculos en las fermentaciones finales con lactosuero de yogur.

El análisis de las diferentes muestras mediante citometría de flujo ha sido llevado a cabo siguiendo una metodología previamente publicada con ligeras modificaciones (Quirós et al., 2007). Una vez centrifugadas las muestras (12.000 x *g* durante 5 min.), la biomasa resultante fue lavada dos veces en tampón fosfato salino (PBS) (pH 7.4, estéril y filtrado por 0,22 μm) y ajustado a una densidad celular de aproximadamente 4 x 10^5 unidades formadoras de colonias por mL en el mismo tampón. Con el fin de teñir las células de *L. casei*, a 200 μL de la suspensiones celulares se les adicionó las soluciones de tinción previamente preparadas. Para los análisis, las muestras fueron teñidas con una mezcla de dos fluorocromos, ChemChromeV6, CV6 (Chemunex, France) e Ioduro de Propidio, IP (Molecular Probes). El CV6 es un éster fluorogénico que difunde al interior celular donde es hidrolizado por las enzimas esterasas intracelulares dando lugar a un producto fluorescente (Cao-Hoang et al., 2008; Parthuisot et al., 2000). La solución stock utilizada fue preparada mediante dilución 1:10 en agua destilada estéril (filtrada por 0,22 μm), de la cual 8 μL fueron añadidos a la suspensión celular y se incubó durante 15 minutos en oscuridad y a temperatura ambiente.

Mientras que el fluorocromo CV6 tiñe las células viables caracterizadas por presentar actividad enzimática, el ioduro de propidio sólo penetra en células que muestran una membrana dañada o permeable. En estas células, el ioduro de propidio penetra uniéndose al DNA formando un complejo fluorescente rojo. La solución stock de ioduro de propidio empleada (1 mg/mL solución en agua, Molecular Probes) fue diluida en agua destilada estéril y añadida a la suspensión celular a una concentración final de 5,25 μg/mL, siendo incubada la mezcla durante 30 minutos en las mismas condiciones anteriores. Cada análisis

fue llevado a cabo en duplicado con el ajuste de compensación correspondiente. Una disposición del densitograma generado (fluorescencia verde versus señal en rojo) al analizar las muestras se muestra en la Figura 3.3.

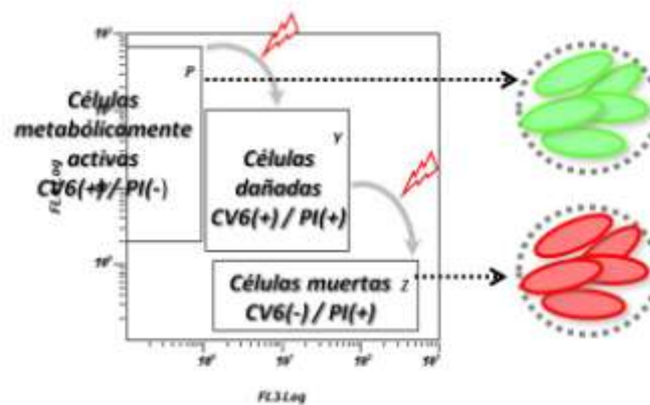


Figura 3.3. Citograma generado durante la tinción dual CV6/IP.

Como controles, se emplearon suspensiones celulares tratadas térmicamente (90ºC durante 30 minutos e inmediatamente enfriadas en hielo), células en fase exponencial y mezclas de células en fase exponencial y tratadas térmicamente (1:1). Asimismo, muestras sin teñir fueron empleadas como controles negativos. Los análisis fueron llevados a cabo en un citómetro de flujo Cytomics FC 500 (Beckman Coulter) equipado con una fuente láser de iones (488 nm) y con cuatro detectores de fluorescencia (Figura 3.4). La fluorescencia procedente de células teñidas con CV6 fue recogida en el canal FL1 (525 nm), mientras que la fluorescencia procedente del IP fue registrada en el canal FL3 (610 nm). Como *standards* internos fueron utilizados 100 µL de microsferas fluorescentes (Perfect Count; Cytognos, España), así en cada análisis fueron adquiridas 2000 microsferas empleando en la adquisición el software Cytomics RXP (Beckman Coulter).

Figura 3.4. Citómetro analizador empleado para la adquisición de datos durante la monitorización fisiológica.

3.2. Producción biotecnológica de ácido lactobiónico

3.2.1. Microorganismo de cultivo

Para la producción de ácido lactobiónico, *Pseudomonas taetrolens* LMG 2336 (obtenida a partir de la Colección Coordinada Belga de Microorganismos, BCCM) fue mantenida a 4ºC en placas de Nutrient Broth Nº3 agar (Fluka), con la siguiente composición: extracto de carne (1 g/L), extracto de levadura (2 g/L), peptona (5 g/L) y NaCl (5 g/L). A partir de estas placas en medio sólido se procedió a revitalizar la bacteria en condiciones aeróbicas.

3.2.2. Preparación del suero lácteo

Se han utilizado dos concentraciones distintas de lactosuero dulce (suministrado por ILAS S.A., Navia, Asturias), sin diluir y lactosuero diluido con agua destilada (en un ratio 1:1 v/v) para seguidamente ajustar el pH a 6,5 con NaOH 6 N. Una vez diluido y con el pH ajustado, el lactosuero fue esterilizado a través de una microfiltración tangencial con un cassette de membranas de PVDF (Millipore, Massachusetts, Estados Unidos) con un tamaño de poro de 0,22 μm.

3.2.3. Condiciones experimentales de cultivo

Se han realizado varias estrategias de bioconversión oxidativa, de este modo, se han realizado experimentos en matraz Erlenmeyer y en biorreactor con control de la agitación y del pH. Así, células procedentes de un cultivo líquido en NB Nº3 fueron empleadas como inóculo del lactosuero (tras centrifugación de la biomasa necesaria a 12.000 x *g* durante 10 minutos) en un matraz Erlenmeyer con capacidad de 500 mL (relación volumen de trabajo:aire, 1:5). Estos cultivos fueron incubados a 30ºC durante 80 h con una agitación de 250 rpm en un incubador orbital (New Brunswick Scientific Co. Inc., Nueva Jersey, Estados Unidos).

Los cultivos en biorreactor se llevaron a cabo en un biorreactor de 2 L (Bioflo 110, New Brunswick Scientific Co. Inc., Nueva Jersey, Estados Unidos) con un volumen de trabajo de 1 L (Figura 3.5). Dicho biorreactor ha estado equipado con un control digital de la unidad, siendo la temperatura fijada a 30ºC y el pH controlado a diversos valores empleando una

disolución concentrada de NaOH como agente regulador del pH. El valor del pH inicial, así como la estrategia de control de pH empleada durante los cultivos han variado según la variable estudiada. Así, se ha estudiado la influencia diferentes valores de pH inicial, control extricto de pH o estrategias de control de pH en fase de producción, variando los valores entre 4,5-7,5 en cada una de las estrategias estudiadas. Igualmente, las tasas de agitación y aireación se fijaron en distintos valores (entre 150-1000 rpm y 0.5-2 Lpm, respectivamente) en función de la variable objeto de estudio (véanse los diferentes subapartados relativos a los resultados de la presente memoria para particularizar). Como agente antiespumante se ha empleado la emulsión diluida (1:10) Y-30 (Sigma-Aldrich, Steinheim, Alemania). Como inóculo de estas bioconversiones en biorreactor se han empleado cultivos en matraz Erlemeyer conteniendo suero lácteo y cultivados con una agitación de 250 rpm y a una temperatura de 30ºC (la duración de los cultivos ha dependido de nuevo en función de la variable sometida a estudio, véase en concreto cada subapartado de resultados para particularizar). Asmismo, la duración (12-48 h) así como el tamaño de inóculo (5-30%) han sido parámetros igualmente estudiados.

Figura 3.5. Equipo de fermentación empleado en los experimentos.

3.2.4. Métodos analíticos

Con el fin de determinar la biomasa, las muestras tomadas a lo largo de los cultivos fueron centrifugadas durante 5 minutos a 16.000 x *g* (Eppendorf, modelo 5415 D, Hamburgo, Alemania), siendo el pellet resultante resuspendido en una solución de NaCl 0,7 % (p/v). El crecimiento celular fue medido espectrofotométricamente como densidad óptica

a una longitud de onda de 600 nm (Shimazdu, modelo UV 1203), siendo estos valores convertidos en términos de biomasa (peso seco, expresado en términos de g/L) empleando la correspondiente curva de calibración previamente determinada.

El contenido en lactosa y ácido lactobiónico procedente de las muestras fue cuantificado siguiendo la misma metodología explicada con anterioridad en el subapartado 3.1.4. En este caso, se emplearon la lactosa (Chem Service, West Chester, Pennsylvania, USA) y el ácido lactobiónico (Sigma-Aldrich, Steinheim, Alemania) como estándares de alta pureza.

3.2.5. Monitorización mediante citometría de flujo

Las muestras tomadas a lo largo de los procesos fermentativos fueron centrifugadas (16.000 x *g* durante 5 min.) con el fin de retirar el sobrenadante y separar la biomasa celular. Previo al proceso de tinción, las células fueron lavadas dos veces en tampón fosfato salino (PBS, pH 7.4, estéril y filtrado por 0,22 µm), para ser sometidas posteriormente a un tratamiento en ultrasonidos durante 2 segundos con el fin de evitar cualquier posible agregación celular previa al análisis multi-paramétrico (Hewitt y Nebe-von-Caron, 2004). Ioduro de propidio (PI, Invitrogen), bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol, $DiBAC_4(3)$, Invitrogen) y carboxifluoresceína (cFDA, Invitrogen) fueron usados como fluorocromos en un protocolo de tinción doble y dual ($DiBAC_4(3)$/PI y cFDA/PI) con el objeto de evaluar el status fisiológico celular (la actividad metabólica, integridad de membrana y polarización de membrana fueron evaluados a través de la tinción con cFDA, PI y $DiBAC_4(3)$, respectivamente). Las soluciones stock fueron preparadas de la siguiente forma: PI fue preparado a una concentración de 1 mg/mL en agua destilada (filtrada por 0.22 µm) y almacenada a 4ºC, mientras que tanto las soluciones de $DiBAC_4(3)$ como las de cFDA fueron preparadas en dimetil sulfóxido (DMSO, Sigma-Aldrich) a una concentración de 1 mM y posteriormente almacenadas a -20ºC. La disolución de tinción de PI fue preparada mediante la dilución de la solución stock en agua destilada estéril, añadiendo dicha solución a la suspensión celular a una concentración final de 5 µg/mL. Esta mezcla fue a continuación incubada bajo condiciones de oscuridad a temperatura ambiente. Las soluciones de trabajo de $DiBAC_4(3)$ y cFDA fueron realizadas en concentraciones de 5 y 10 µM en PBS conteniendo 1 mM EDTA, respectivamente. Las células teñidas con fueron posteriormente incubadas con 1 µM $DiBAC_4(3)$ o 0.1 µM cFDA durante 15 min en oscuridad y a temperatura ambiente.

La adquisición de los correspondientes datos multi-paramétricos fue llevada a cabo en un citómetro analizador Cytomics FC 500 (Beckman Coulter) equipado con una fuente de excitación laser de 488 nm y 633 nm procedente de un laser de argón. La fluorescencia verde de las muestras (correspondiente a células teñidas con $DiBAC_4(3)$ y cFDA) fue

recogida en el canal FL1 (530 nm), mientras que la fluorescencia de PI fue registrada en el canal FL3 (610 nm). Una disposición del citograma generado se muestra en la Figura 3.6. Cada análisis fue realizado por duplicado con una velocidad de adquisición de 4000 eventos/seg. La adquisición se llevó a cabo con el software Cytomics RXP software (Beckman Coulter). Tanto las ventanas como cuadrantes fueron establecidas en base controles de tinción. En cada análisis dual-paramétrico DiBAC$_4$(3)/PI y cFDA/PI, 150.000 y 100.000 eventos fueron recogidos, respectivamente. Siendo analizados los datos a través del software Summit v4.3 (DakoCytomation, Colorado, USA).

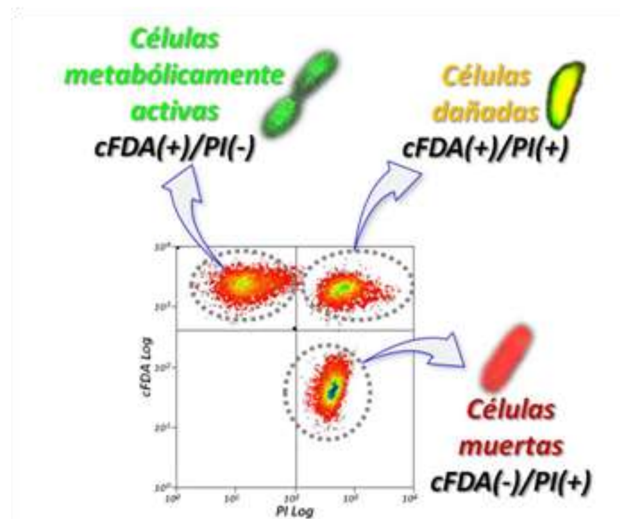


Figura 3.6. Densitograma o citograma donde se muestran la diferentes subpoblaciones generadas con la aplicación del protocolo de tuición cFDA/IP

3.2.6. Separación celular

La separación celular (*Fluorescence-Activated Cell Sorting*, FACS) fue realizada en un MoFlo XDP *cell sorter* (DakoCytomation) equipado con un laser de argón que emite luz azul a longitudes de onda de 488 y 630 nm (200 mW) (Figura 3.7). La fluorescencia verde procedente de las células teñidas con cFDA fue detectada en el canal FL1 (530 nm), mientras que la fluorescencia roja de la señal de PI fue recogida en el canal FL3 (610 nm). Microesferas fluorescentes (Flow-check Pro Fluorospheres, Beckman Coulter) con tamaños de 10, 6 y 3 µm fueron usadas para evaluar la hidrodinámica del sistema y el alineamiento óptico del citómetro (coeficiente de variación, valor CV, alrededor de 2%). Las muestras fueron separadas en modo *purify* con una velocidad alrededor de 200 células/seg. empleando Isoton II (Beckman Coulter) como solución fluídica de transporte. El sistema

operó con una presión de 54 psi y fue equipado con un tamaño de *nozzle* de 70 µm. Las ventanas de separación fueron establecidas en base a la fluorescencia del canal FL1 (células cFDA(+)/PI(-)) frente a la fluorescencia del canal FL3 (células cFDA(-)/PI(+)). La adquisición de datos fue realizada usando el software Summit v5.0 (DakoCytomation). Las células procedentes de las diferentes subpoblaciones discriminadas en función de los cuadrantes (véase Figura 3.6) fueron separadas (96 spots) en placas de agar conteniendo lactosa (10 g/L), peptona (2 g/L), extracto de levadura (1 g/L), K_2HPO_4 (0.2 g/L), azul de bromofenol (0.08 g/L) y agar (15 g/L) con el objetivo de comprobar tanto la capacidad oxidativa como la viabilidad de las mismas. Dichas placas fueron incubadas a 30ºC durante 96 h.

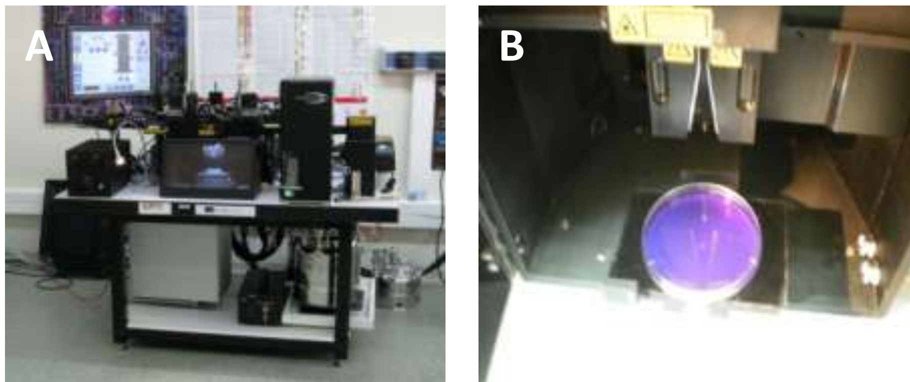
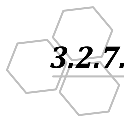
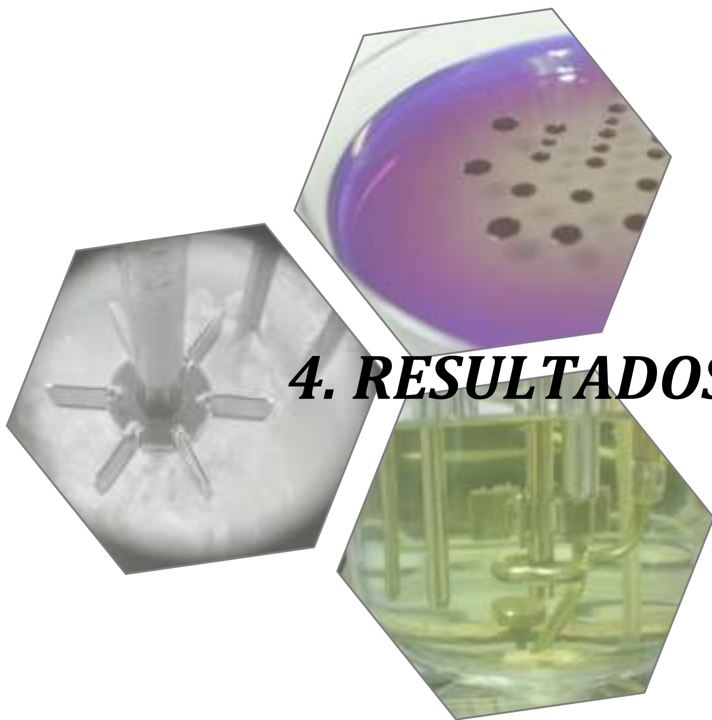


Figura 3.7. Citómetro separador empleado en los experimentos (A). Detalle de la disposición de la bandeja de separación (contiene una placa de agar sobre la que se han separado las células en 96 *spots*) con las placas deflectoras dentro de la cámara hermética (B).



3.2.7. Microscopía confocal de fluorescencia

Las muestras teñidas fueron también examinadas en un microscopio laser confocal Leica TCS-SP2-AOBS (Leica Microsystems Inc., Heidelberg, Alemania) a unas longitudes de excitación de 488 y 568 nm, con una longitud de emisión de 530 (fluorescencia verde) o 630 nm (fluorescencia roja).



4. RESULTADOS

4. RESULTADOS

4.1. Producción de ácido láctico por Lactobacillus casei

En este subapartado se recogen los resultados referentes a la obtención biotecnológica de ácido láctico a partir de lactosuero de yogurt empleando *L. casei* como plataforma microbiana. Como se ha venido señalando en anteriores subapartados (2.2), el ácido láctico es un ácido orgánico maduro cuya producción biotecnológica está más que asentada en el ámbito industrial. Sin embargo, la industria y el mundo académico continúan aunando esfuerzos en la búsqueda de nuevas fuentes que sirvan como materia prima o sustrato de la fermentación ácido-láctica. Ante las enormes perspectivas de crecimiento del ácido poliláctico, las actuales tendencias en investigación relacionadas con el ácido láctico se han focalizado en la búsqueda y el empleo de nuevos sustratos ricos en carbohidratos fermentables. En la presente memoria se ha propuesto al lactosuero de yogurt, procedente de yogures caducados o retirados de la cadena comercial, como una matriz idónea para la obtención de ácido láctico. La industria láctea estima que alrededor del 1% del volumen total de yogures comercializados (categoría en la que se incluyen todos los postres fermentados) han de ser retirados y ser tratados como un residuo, con el consiguiente coste económico. En el artículo de investigación que a continuación se presenta, se ha hecho hincapié en la importancia de la naturaleza de los azúcares y de cómo la presencia de estos carbohidratos lleva consigo la modulación del catabolismo de los mismos por parte de *L. casei*. Asimismo, se ha estudiado la importancia de cómo diferentes parámetros operacionales influyen sobre el rendimiento y la productividad del sistema fermentativo.

En suma, se ha propuesto al lactosuero de yogurt como sustrato dentro de un sistema caracterizado por no precisar de pretratamiento ni sacarificación alguna a diferencia de otros residuos empleados como sustratos, caso de los residuos lignocelulósicos o agro-industriales.

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Residual yoghurt whey for lactic acid production

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ABSTRACT

The operation in global yoghurt market provokes frequently the reject and withdrawn of yoghurt derivates out of shelf life. Yoghurts that have past their ‘best before’ date constitute a waste that has to be environmentally treated. Its use as a source for lactic acid production by *Lactobacillus casei* is studied in this work, being also a proposal that can reduce the end-of-cycle impact of the residue. Production of yoghurt generates a residue highly supplemented with sugar and fruits syrups that can be metabolized to lactic acid, but no data are available in the literature about this potential transformation into a commercial valuable lactic acid. Bioconversion of total sugars around 44% was achieved in pH controlled batch fermentation after 34 h. Control of pH and yeast extract supplementation in the preculture stage have shown a remarkable enhancement of fermentation performance due to the reduction in the toxicity and the increase in nitrogen content. Successive bioconversions of lactose, glucose and sucrose have been shown, with a sugar consumption profile giving a hierarchical mode consumption in the order glucose > sucrose > lactose.

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1. Introduction

Over the past years, yoghurt and fermented dairy products have been converted in the fastest-growing dairy segment, contributing more and more with higher rates to global sales. Recent forecasts from consulting companies even consider the increase of global sales over the coming years, especially in emerging markets as Asiatic area. This product has grown at a rate of double-digit for the last years, so world market of liquid yoghurt had a rate of growth between 13 and 18% from 2000 to 2006 [1].

The future of yoghurt production, considering the environmental implications of increasing manufacture, involves the treatment of the dairy fermented products out of shelf life. The withdrawn and rejects from market of damaged yoghurts and drinking yoghurts or those over their sell-by date, create an important amount of human foodstuff waste which is unsuitable for sale, although in some cases it could be used for animal feed [2]. Indeed, dairy industry estimates that around 1% of total yoghurt production including not only rejected and expired yoghurts but also yoghurts generated from overstocks and product line changeover are wasted off. To date, yoghurt residuals are employed in animal feeding with other rest-streams generated by food industry. However, their high water content limits their use as animal feed, making convenient to remove liquid whey. Whereas dry fraction is used for animal feed purposes, yoghurt whey is proposed here to be used as source for lactic acid fermentation, the main challenge of this work.

Yoghurt is a dairy product sweetened with high levels of conventional added sugars such as sucrose and glucose [3]. For this reason their environmental impact is higher than cheese whey and some additional efforts must be carried out to avoid its waste. The most noteworthy feature of this residue is the high content on fermentable sugars such as lactose, glucose and sucrose. Therefore, one of its possible uses is to be

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considered as raw material in fermentations in order to reduce its waste with substantially lower environmental impacts emphasizing the sustainable concept in the food industry. Nevertheless, to date no studies have been focused on the biotechnological use of residual yoghurt whey.

Lactic acid and its derivates are widely used in several sectors as food or pharmaceutical industries. Moreover, recently there is an additional and increasing interest on lactic acid to be used for polylactic acid (PLA) production. This sustainable polymer made from renewable resources is a substitute for petrochemical-based polymers, an environmental-friendly biodegradable plastic material [4]. Different renewable resources and agroindustrial wastes have been used as fermentative substrate for lactic acid production, such as corn [4], starch [5], rice bran [6], wheat flour [7] or date juice [8]. However, their potential use is limited by costly and laborious steps such as enzymatic hydrolysis or simultaneous saccharification [9]. Furthermore, the use of yoghurt whey as substrate for fermentation reduces the wasteful and costly disposal of unsuitable yoghurts for human consumption, advancing in its chain value.

Yeast extract adds suitable nutrients like minerals, vitamins and peptides that promote optimum growth of lactic acid bacteria in low nitrogen nutrient media. An efficient bacterial growth is promoted by the presence of growth factors in yeast extract, although it is strongly influenced not only by the kind of autolysate but also by culture medium composition [10]. Yeast extract are usually obtained from baker's or spent brewer's yeast by autolysis [11]. However, this complex nutrient presents a high cost, which becomes a major shortcoming in fermentation processes. Moreover, some food by-products are able to be considered as appropriate supplements such as fish hydrolysates, spent brewer's or cider's yeast lees, an attractive replacement of yeast extract in fermentation processes [8,12].

The aim of this research work was to evaluate the potential use of yoghurt whey as source for lactic acid production by *Lactobacillus casei* through an eco-friendly process. The yoghurt whey was used as a feedstock in fermentations, so different strategies were performed either with or without pH control. In order to enhance fermentation, preculture supplementation with yeast extract was also tested. Likewise, another aim of this study was to find out the sugars consumption profile by *L. casei* throughout the fermentation processes in order to determine the efficacy and the sequence of substrates consumption. By this means, lactic acid production and sugar consumption was monitored by HPLC throughout the fermentation experiments carried out.

2. Materials and methods

2.1. *Microorganism*

The *L. casei* ATCC 393 strain, obtained from the American Type Culture Collection (Virginia, USA), was maintained frozen (in 40% v/v glycerol at −20 °C). The strain was cultured on MRS broth (de Man Rogosa and Sharpe, Biokar Diagnostics, France) or in solid plates (containing 2% w/w agar).

2.2. *Yoghurt whey preparation*

Yoghurts expired date were previously mixed in order to obtain yoghurt whey by heat treatment (thermal coagulation) removing fat and caseins. Next, the remaining fat was withdrawn from yoghurt whey by centrifugation at 12,000 × gravity for 10 min at 4 °C. Dilution was performed with distilled water (1:1) and adjusted to pH 6.5 (by adding NaOH 1 mol dm^{-3}). Once diluted, yoghurt whey was sterilized using a tangential microfiltration device equipped with a PVDF membrane-cassette (Millipore, Massachusetts, USA) 0.22 μm pore size. Table 1 summarizes the features of yoghurt whey.

2.3. *Culture and fermentation conditions*

The methodology employed on fermentation experiments is summarized in Fig. 1. After reactivation on MRS under microaerophilic conditions (without shaking), 10% (v/v) of this culture (exponential growth phase) was used to inoculate either 90 mL of yoghurt whey or yoghurt whey supplemented with 2.5 g L^{-1} of yeast extract as precultures. Precultures were incubated at 37 °C for 20 h, at 100 r.p.m in an orbital shaker (Infors HT, model Flyer Aerotron, Bottmingen, Switzerland). Subsequently, these precultures were employed to inoculate yoghurt whey batch fermentations. Uncontrolled pH fermentations were performed in a final working volume of 1 L at 37 °C in an orbital shaker (100 r.p.m). Whereas, batch experiments with pH controlled were performed in a 2 L bioreactor (Bioflo 110, New Brunswick Scientific Co. Inc., NJ, USA) with a final working volume of 1 L, equipped with a digital control unit; temperature was controlled at 37 °C and the pH was set at 6.5 using NaOH 3 mol dm^{-3}, agitation was set at 50 r.p.m to keep fermentation medium mixed. Samples were collected at regular intervals of 2 h to determine optical density and pH; cell-free supernatants (0.45 μm filtered) were stored frozen (−20 °C) until analysis. Fermentations were carried out in duplicates as two independent experiments.

Table 1 – Characterization of yoghurt whey used in fermentation experiments.

	TKN (mg L^{-1})	Protein concentration (g L^{-1})	Density (kg m^{-3})	°Brix	Moisture (%)	COD (g L^{-1})	pH (units)
Raw yoghurt whey	580	3.6	1042	13	91.6	90	4.2
Yoghurt whey medium for fermentation experiments	309	1.9	1018	6.5	96.1	65	6.5

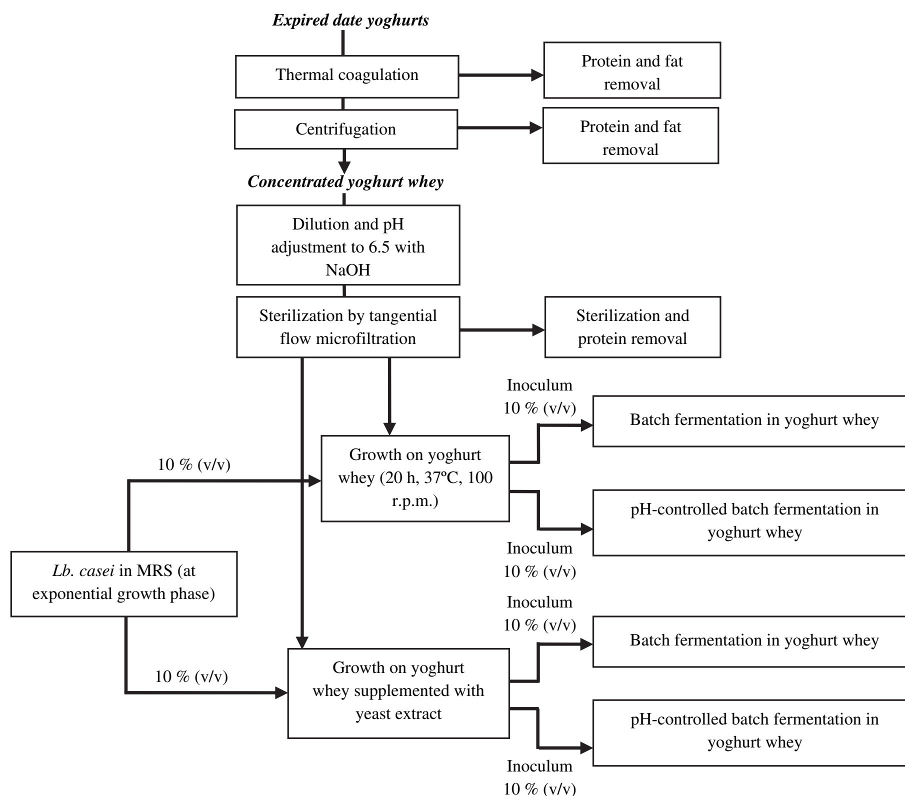

Fig. 1 – Diagram that summarizes the processes to use residual yoghurt whey for lactic acid production as carried out in this paper.

2.4. *Bacterial growth*

Culture samples were centrifuged for 10 min at 16,000 × gravity and subsequently the pellet was resuspended in NaCl 0.7% (w/v). Bacterial growth was measured by optical density at 660 nm (Shimazdu, UV 1203 model). Optical density data was converted to cell dry weight using the corresponding calibration curve previously obtained, and results were expressed as $g\,L^{-1}$.

2.5. *Analytical methods*

Protein content was determined according to Bradford [13] (Sigma–Aldrich reagent, Steinheim, Germany) using bovine serum albumin as standard.

Lactose, glucose, sucrose and lactic acid concentrations from fermentation broth were measured by HPLC according to a method described by Preduzzi et al. [14] with minor modifications. The setup used for the analysis was a chromatograph Agilent, 1200 Series model (California, USA) with an ICSep ICE-ION-300 column (Transgenomic Inc., San Jose, California, USA). The following analytical conditions were used: 450 mM H_2SO_4 as mobile phase (pH 3.1), column temperature set at 75 °C and flow rate of 0.3 mL min^{-1} with the Refractive Index as detector. Samples were quantified according to analytical external standards with HPLC grade; in that way, lactose, sucrose and lactic acid were obtained from Chem. Service (West Chester, Pennsylvania, USA), glucose from Merck (Darmstadt, Germany) and ethanol from Panreac (Barcelona, Spain). Ethanol was not detected since *L. casei* is a facultative heterolactic fermentative microorganism, although lactic acid is the dominant end product under typical fermentation conditions. Data acquisition and analysis were performed with Agilent ChemStation software.

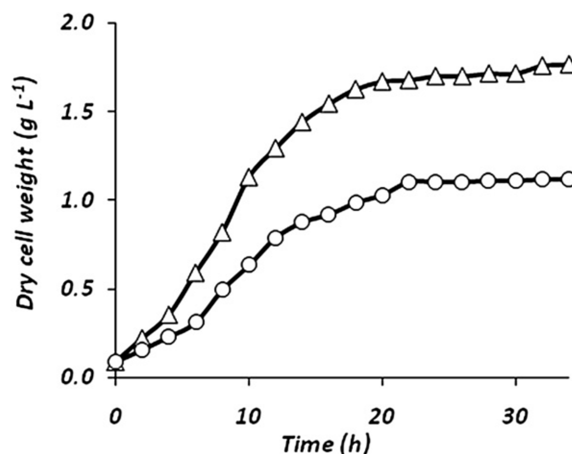

Fig. 2 – *Lactobacillus casei* growth evolution in residual yoghurt whey cultures with (△) and without yeast extract supplementation (○).

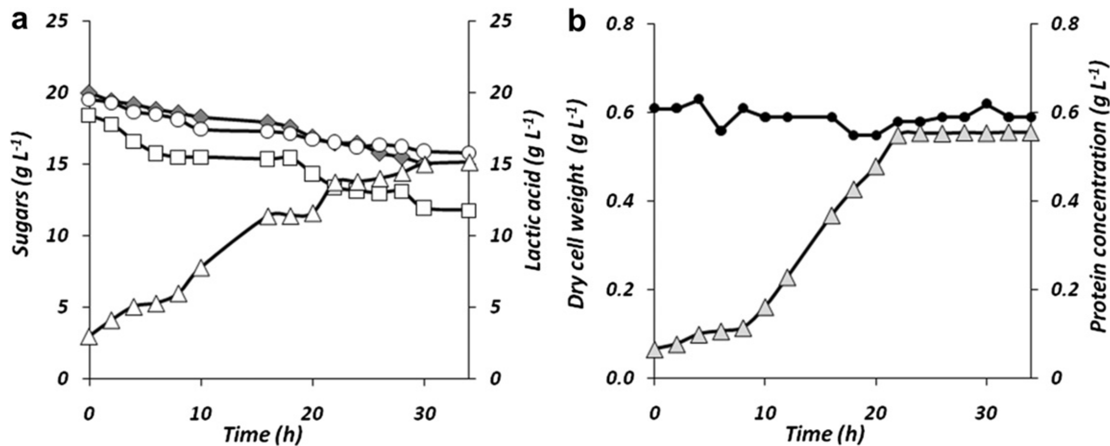

Fig. 3 – (a) Lactose (◆), sucrose (○) and glucose content (□), as well as lactic acid formation (△) through a pH uncontrolled fermentation without yeast extract supplementation in preculture stage. (b) Biomass (expressed as dry cell weight) (▲) and protein (●) concentration of pH uncontrolled fermentation without yeast extract.

3. Results and discussion

Batch fermentation is usually used for lactic acid production at industrial scale. Previous to the fermentation process, the raw yoghurt whey and the yoghurt whey sterilized (by filtration) were characterized. The main parameters are summarized in Table 1. Since yoghurt whey presents a high content in sugars, it is a suitable substrate to obtain a value-added product such as lactic acid.

3.1. *Effect of yeast extract supplementation in residual yogurt whey on bacterial growth*

It is well known in fermentation studies that supplementation of proteins and nitrogen in general improves the bioconversion of the substrates. In order to evaluate its effect on the growth of the lactic acid bacteria strain in the residual yogurt whey, commercial yeast extract was used. Fig. 2 shows *L. casei* growth evolution in residual whey cultures, with or without yeast extract supplementation. In the conditions tested, cells reached exponential phase after 20 h of incubation. In the supplemented medium, higher cell densities were obtained (around 1.8 $g\,L^{-1}$ versus 1.2 $g\,L^{-1}$ without supplementation).

The influence of yeast extract on lactic acid bacteria growth and on lactic acid yield has been verified by several works [10,11,15]. The peptide content from yeast extract may be positive since peptides and peptones stimulate all parameters involved in fermentation [9]. The possibility of using hydrolysed whey protein as supplementation has also been investigated obtaining good lactic acid productivity, yield and high lactose consumption [15].

At laboratory scale yeast extract is usually utilized in fermentation processes as supplementation [16]. Usually the problem to perform medium supplementation is the high cost of the protein supplement that makes the process economically unfavourable. From an industrial practical approach, the culture medium should be easy to prepare and at low cost.

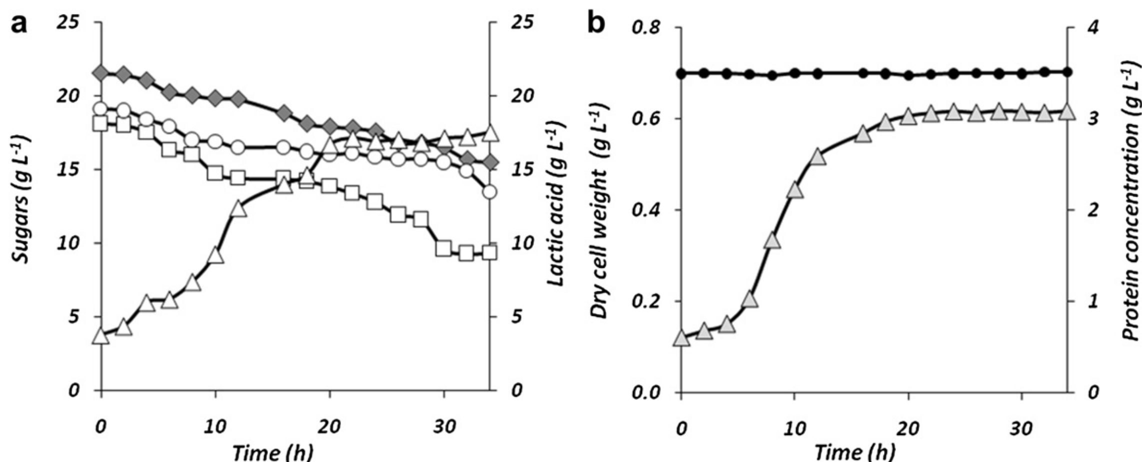

Fig. 4 – (a) Lactose (◆), sucrose (○) and glucose content (□), as well as lactic acid formation (△) through a pH uncontrolled fermentation with yeast extract supplementation in preculture stage. (b) Biomass (expressed as dry cell weight) (▲) and protein (●) concentration of pH uncontrolled fermentation with yeast extract supplementation in preculture.

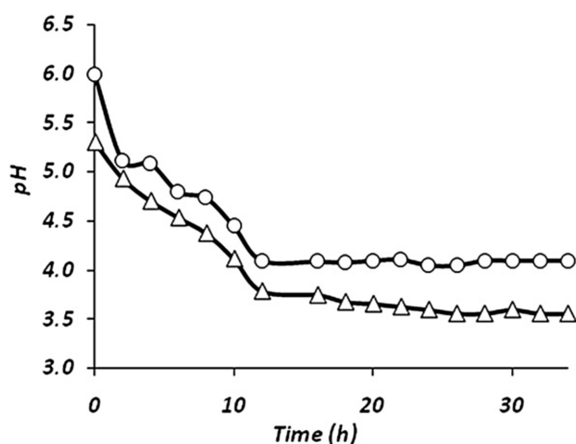

Fig. 5 – pH evolution in batch pH uncontrolled fermentations, with (△) and without yeast extract supplementation in preculture stage (○).

Moreover, addition of supplements would increase the impurity level in the fermentation broth which has to be separated for further product purification. For these reasons and based on the results obtained (Fig. 2) the potential capacity of the residual yogurt whey for lactic acid production was investigated without the use of costly nutrients during batch fermentation. In this work, the advantage of using supplements as yeast extract only in the preculture stage was tested, in order to compare the results obtained.

3.2. Fermentation without pH control

In accordance with the results shown in Fig. 3, the pH uncontrolled fermentation proceeded slowly. A relatively high content of residual sugars were left on the fermentation broth leading to a low yield.

Yeast extract supplementation in the preculture stage (Figs. 3 and 4) had a positive effect on fermentation performance. The lag phase was clearly reduced and at the same time the lactic acid production associated to bacterial growth was increased.

After 34 h of fermentation, the quantity of lactic acid obtained from both experiments was 14.5 and 12.25 g L^{-1} for pH uncontrolled batch culture with and without yeast extract supplementation in preculture stage respectively. Sugar consumption was more efficient in the case of fermentation with yeast extract supplementation in precultures since it achieved remaining final concentrations of 15.5 g L^{-1} of lactose, 13.47 g L^{-1} of sucrose and 9.32 g L^{-1} of glucose. On the other hand, the remaining total sugar content without yeast extract was higher, 42.54 g L^{-1} versus 38.28 g L^{-1} at the end of the fermentation processes.

Differences in the pH values were observed in uncontrolled and controlled fermentations, as shows Fig. 5. Whereas fermentation without yeast extract reached a pH of 4.2 at 12 h, the fermentation with yeast extract in preculture stage fell under pH 4, maintaining the pH near 3.5. The different evolution suggests a favourable and positive effect of yeast extract supplementation in the preculture stage, although the behaviour is very similar within the first 12 h of the process. Differences in the initial pH values were in accordance with the acidity reached in the preculture. Hence, as it is shown in Fig. 5 the pH inhibition and the low cell growth provoked the inability of the strain in using the remaining sugars. As the fermentation continued, the rate slowed down because of accumulation of lactic acid so pH values fell under 4.2 causing metabolic inhibition.

The lactic acid production of uncontrolled pH fermentation was around 14 g L^{-1}. Other authors have found productions of 10 g L^{-1} of lactic acid from fermentation of cheese whey without pH control using *Lactobacillus helveticus* [17]. The pH inhibition and the final lactic acid concentration were suggested to have a strong inhibitory effect.

Regarding to total protein content in fermentation broth, it can be concluded that this parameter had not influence in the global fermentation profile since the initial concentration was maintained.

3.3. Fermentation with pH control

Figs. 6 and 7 show that *L. casei* consumes sugars at similar rates, but in this case the higher lactic acid production, around

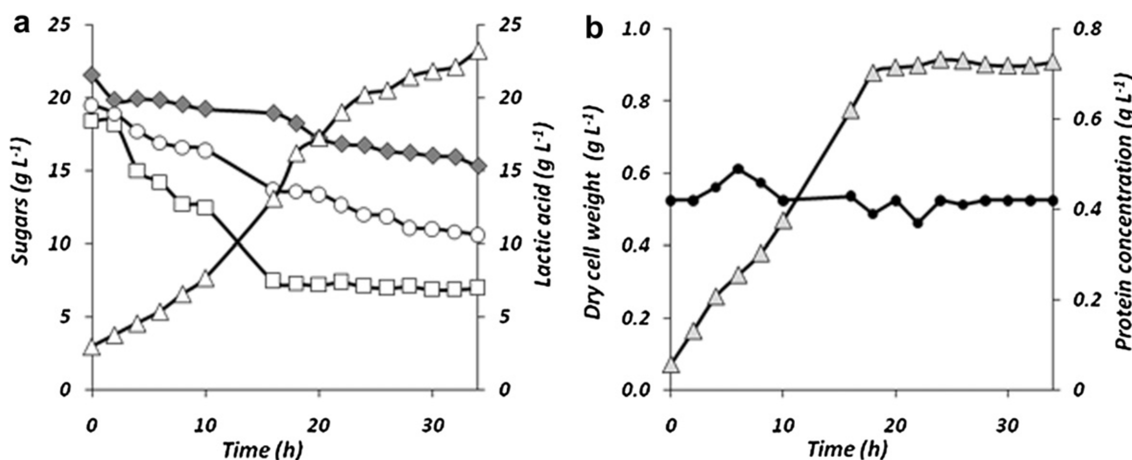

Fig. 6 – (a) Lactose (◆), sucrose (○) and glucose content (□) and lactic acid formation (△) through a pH controlled fermentation in bioreactor (37 °C). (b) Biomass (▲) and protein (●) evolution throughout this fermentation.

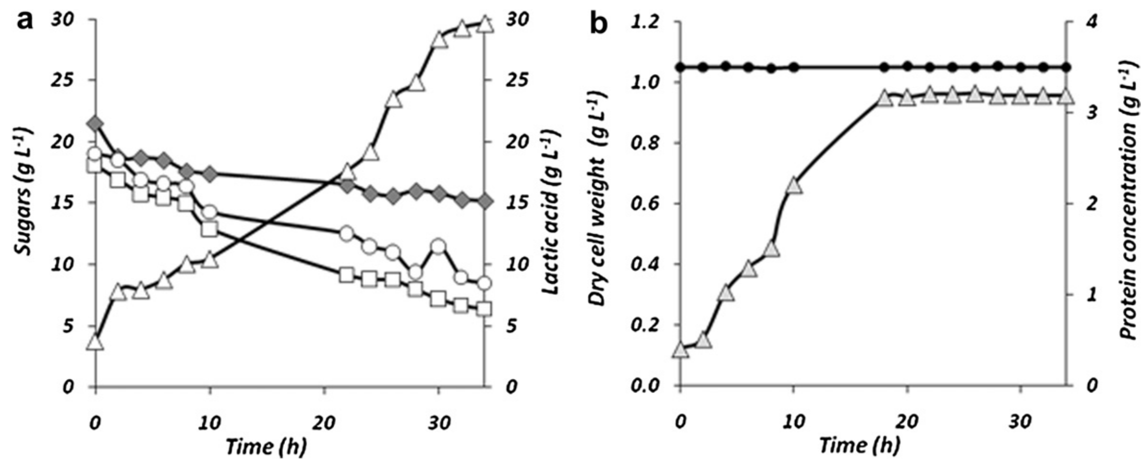

Fig. 7 – (a) Lactose (◆), sucrose (○) and glucose content (□) and lactic acid formation (△) through a pH controlled fermentation with yeast extract supplementation in preculture stage. (b) Biomass (▲) and protein (●) evolution throughout this fermentation.

23 g L^{-1} (Fig. 7), was linked to a better consumption of glucose and sucrose versus lactose. Protein content has not changed throughout fermentations, both in pH-free and controlled batch processes. As mentioned, the possible effect observed for yeast extract supplementation in preculture stage may be probably related to its complex composition. The undefined components of yeast extract, also rich in vitamins (specially the B group) showed growth-promoting properties on lactic acid bacteria as reported [10].

The results (Figs. 6 and 7) show how the glucose and sucrose were metabolized quickly in comparison to lactose. Nevertheless, remaining sugars were still present in fermentation broth at the end of the experiments; approximately 15 g L^{-1} of lactose remains in all experiments. The lactic acid production suffered a significant increase at late stationary growth phase and the clearly growth and productivity enhancement surged as result of yeast extract supplementation in the preculture stage, and therefore it meant a better efficiency of the biomass reached (0.97 g L^{-1}) over the process yield. The content of residual sugar varied from 15.2 g L^{-1} of lactose to 8.5 g L^{-1} of sucrose, whereas in the same fermentation procedure but without yeast extract supplementation the final sugar values were 15.3 g L^{-1} of lactose, 7 g L^{-1} of glucose and 10.6 g L^{-1} of sucrose. Besides, total protein content has been demonstrated not to be a key parameter of the process since their concentration did not suffer any modification.

The carbohydrate consumption by *L. casei* reflects its ability to efficiently use carbon sources available in yoghurt whey. Figs. 6a and 7a contain meaningful information concerning the performance of carbohydrate metabolism by *L. casei*. These results suggest a co-metabolic biotransformation by *L. casei*, since this strain is able to metabolize actively glucose, lactose and sucrose. However, there is a preference of carbohydrate consumption, thus, glucose is the first sugar metabolized, whereas sucrose and lactose content slightly decreased over time. Experimental results demonstrated that *L. casei* prefers glucose and sucrose over lactose as primary carbon and energy source throughout lactic acid production processes from yoghurt whey. Carbohydrate consumption curves followed nearly the same pattern, but in each case the amount of lactic acid produced was diverse due to the different sugar consumption. Moreover, fermentation of carbohydrates is achieved sequentially; this suggests hierarchical simultaneous sugar utilization with a control accomplished by a regulatory mechanism.

Consequently, lactose is transported through the cytoplasmic membrane by means of a permease as lactose phosphate, followed by cleavage by β-galactosidase to yield glucose and galactose. Thereby, glucose and galactose are phosphorylated by glucokinase and metabolized through the glycolytic pathway. Sucrose fermentation is mediated by a sucrose phosphotransferase system, which is simultaneously cleaved by a sucrose hydrolase to yield glucose and fructose [18,19].

A conversion rate of 44% from initial total sugar to lactic acid was obtained in the best fermentation experiment, which

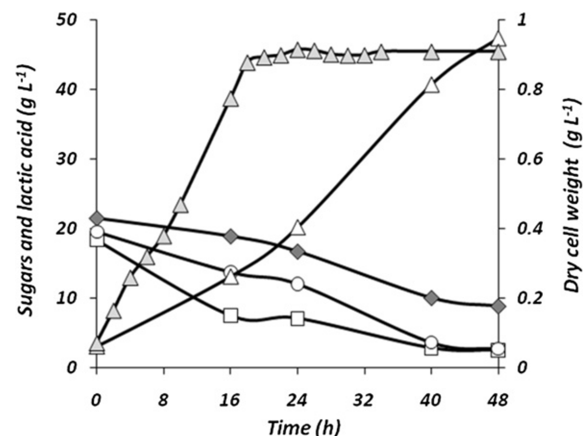

Fig. 8 – Experimental values obtained corresponding to pH controlled fermentation without yeast extract in preculture stage. Evolution of biomass (▲), lactose (◆), sucrose (○) and glucose content (□), as well as lactic acid formation (△) throughout batch culture.

Table 2 – Summary of values obtained in the yoghurt whey fermentation experiments.

	Fermentation without pH control	Fermentation without pH control and with yeast extract in preculture stage	Fermentation with pH control	Fermentation with pH control and with yeast extract in preculture stage
Initial lactose ($g L^{-1}$)	21.5	21.5	21.5	21.5
Initial glucose ($g L^{-1}$)	18.4	18.1	18.4	18.1
Initial sucrose ($g L^{-1}$)	19.5	19.1	19.5	19.0
Final lactose ($g L^{-1}$)	15	15.5	15.3	15.2
Final glucose ($g L^{-1}$)	11.78	9.32	7	6.4
Final sucrose ($g L^{-1}$)	15.76	13.47	10.6	8.5
Lactic acid produced ($g L^{-1}$)	12.25	14.5	20.2	25.9
Lactic acid yield on substrate ($g g^{-1}$)	0.72	0.71	0.76	0.9
Lactic acid productivity ($g L^{-1} h^{-1}$)	0.36	0.426	0.59	0.76

proceeded without any nutrient adding in batch fermentation. Although experiments performed showed an acceptable lactic acid production, a remarkable enhancement in conversion yield was obtained with yeast extract supplementation in the preculture stage. In comparison with the same fermentation procedure but without yeast supplementation in precultures, the bioconversion diminished until 34%. Thereby, yoghurt whey has been shown as a favourable and suitable medium for lactic acid production. Another important aspect is the *L. casei* ability to consume nearly all remaining sugars left in broth after 34 h of culture. It can be inferred observing the carbohydrate consumption patterns that *L. casei* presents different yields of lactic acid on sugar substrate, being the higher related with glucose and sucrose. A higher performance and fermentation efficiency could be achieved if fermentation time is increased up to 48 h obtaining a bioconversion rate over 75% as can be observed in Fig. 8 for pH controlled fermentation and without yeast extract supplementation in preculture stage.

Table 2 summarizes the fermentation experiments carried out in this work. As it is shown, supplementation with yeast extract in preculture stage and controlled pH promote lactic acid production. Moreover, it can be seen that highest lactic acid is reached with pH controlled conditions, whereas fermentation with yeast extract in preculture stage rendered better productivity. The maximum lactic acid concentration obtained was 25.9 $g L^{-1}$ corresponding to batch fermentation with pH control and yeast extract supplementation in preculture stage. Whereas, fermentations without pH control and without yeast extract supplementation in preculture stage were performed with a final value of 12.25 $g L^{-1}$ in lactic acid and with a lactic acid yield on substrate of 0.72 $g g^{-1}$. Comparing with the uncontrolled pH fermentation, the pH control showed 64.8% improvement in lactic acid production. Yeast extract supplementation in preculture stage showed an improvement of 28% in lactic acid production in pH controlled cultures.

Yeast extract favoured to enhance lactic acid maximum production, increasing the process yield. The lack of nitrogen supplementation (either yeast extract or peptones) in the medium at the preculture stage induces an increase in lag phase length of the culture as a result of the cellular adaptation to the whey [20].

Finally, with the aim to determine the environmental impact, chemical oxygen demand (COD) can be used as an indirect measurement of soluble and insoluble organic matter. The organic waste reduction can be achieved as result of microbial pure and mixed cultures in food by-products such as cheese whey [21]. In this work, COD was analyzed before and after fermentation experiments in order to determine the theoretical reduction of the pollution. As it seen in Table 3, it can be concluded that fermentation process reduced the environmental impact (COD values dropped from 90.4 to 72.5 $g L^{-1}$) although the increase on initial values were result of yeast extract supplementation and the necessary use of MRS on previous preculture steps, maybe explained by the use of high organic content in precultures used as inoculum.

Table 3 – Chemical oxygen demand values of yoghurt whey throughout process.

Medium	COD ($g L^{-1}$)
Initial yoghurt whey	90
Yoghurt whey after tangential filtration and before fermentation experiments	65
Yoghurt whey after fermentation experiments without cells	72

4. Conclusions

Yoghurt whey, a waste material containing among other constituents important concentrations of lactose, glucose and sucrose, added in the yoghurt production process, has been revealed as a suitable and direct substrate to lactic acid production by *L. casei* ATCC 393. An adequate pH control has shown to produce an important enhancement in carbohydrate bioconversion efficiency. In addition, microbial carbohydrate uptake and lactic acid production have been improved by the presence of surplus growth factors from yeast extract in preculture stage. The evolution of the sugar content has shown how glucose is metabolized more efficiently by *L. casei*, and then how lactose and sucrose are consumed at similar rates. All sugars are metabolized at

different stages, already from the initial fermentation time. Results suggests that *L. casei* was able to metabolize most glucose and sucrose within 34 h to produce 25.9 g L^{-1} lactic acid, but the lactose content left was 15.2 g L^{-1}. In spite of the lactic acid levels achieved, residual sugar content could be further reduced. Alternative fermentation strategies could be used to improve the efficiency compared with the traditional batch culture avoiding inhibition effects. The results obtained here supported the interest in the biotechnological re-use of yoghurt-expired date whey, otherwise treated as a waste. In brief, lactic acid production from a raw material as yoghurt proved to be effective through glucose, sucrose and lactose bioconversions. Thereby, it constitutes a sustainable and interesting bioprocess to obtain a value-added industrial product from an accepted worthless feedstock until now.

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4.2. *Monitorización fisiológica de Lactobacillus casei*

En este subapartado se ha monitorizado fisiológicamente la fermentación ácido-láctica llevada a cabo por *L. casei* con el objetivo de producir ácido láctico a partir de lactosuero de yoghurt. Así, se ha evaluado la influencia de diferentes condiciones operacionales y del propio microambiente sobre la fisiología de *L. casei*. En este artículo se describe el comportamiento del sistema bajo condiciones de presencia o ausencia en el control del pH y de cómo la respuesta fisiológica de *L. casei* se ve influenciada por estas condiciones. A diferencia de los sistemas fermentativos clásicos donde la acidificación puede significar la pérdida de viabilidad, *L. casei* desarrolla en este caso una robustez fisiológica ante condiciones desfavorables en contraposición con la respuesta fisiológica exhibida por el microorganismo ante condiciones *a priori* mucho más favorables (bajo control de pH), donde el lactato parece jugar un papel clave en la respuesta fisiológica. Bajo estas últimas condiciones, *L. casei* experimenta una fuerte pérdida de la viabilidad celular, siendo las subpoblaciones de células dañadas y muertas las mayoritarias en tiempos de fermentación relativamente prolongados. En el trabajo se ha utilizado a la citometría de flujo multi-paramétrica como herramienta en la monitorización y control de dichas respuestas fisiológicas. Tanto la actividad metabólica como la integridad de la membrana han sido las propiedades físiológicas evaluadas a lo largo de los sistemas fermentativos llevados a cabo, con el fin de proponer las condiciones operacionales más adecaudas desde el punto de vista fisiológico de *L. casei*. Estos resultados han demostrado la importancia de las condiciones operacionales de cultivo sobre la viabilidad celular de *L. casei*, circunstancia que puede llevar a un desaprovechamiento de la capacidad metabólica del microorganismo en fermentaciones prolongadas en el tiempo.

Publicación: Physiological states of *Lactobacillus casei* fermentation in a mixed natural substrate with lactose, glucose and sucrose.

Situación: En elaboración.

Physiological states of *Lactobacillus casei* fermentation in a mixed natural substrate with lactose, glucose and sucrose

Abstract

Although *Lactobacillus casei* is well-known as a potential lactic acid-producer microorganism with substantial interest on industrial sphere, the influence of different bioprocessing conditions on its cellular functionality and physiological status is not yet studied. In the present study, physiological states of *L. casei* during a sugars-to-lactic acid bioprocess using residual yoghurt whey as inexpensive source were monitored by multiparameter flow cytometry. *L. casei* displayed a robust physiological status under uncontrolled-pH conditions despite the acid microenvironment encountered (pH<3.6), suggesting the development of an acid tolerance response. Conversely, a stress-induced physiological response took place when a controlled-pH cultivation strategy was adopted. The decline in metabolically active subpopulation revealed that *L. casei* cells entered into a state of cellular damaged due to stressful bioprocessing conditions encountered at stationary growth phase. Such conditions were featured by a high lactate titer (41.5 g/L) which was deleterious to *L. casei* cells. Regardless the physiological response, a lactic acid titer of 41.5 g/L with a yield of 0.91 g/g on sugars and a volumetric productivity of 0.86 g/L.h were achieved under controlled-pH conditions. The sugar pattern metabolism with simultaneous co-fermentation of glucose, sucrose and lactose was additionally maintained despite either the stress-induced physiological or the acid tolerance responses developed by *L. casei* cells. These results provide further understanding on the influence of acid and non-acid bioprocessing conditions on the physiological responses of *L. casei*, suggesting additionally the feasibility of glucose, sucrose and lactose co-fermentation as substrates for lactic acid production.

Keywords: multiparameter flow cytometry; lactic acid; *Lactobacillus casei*; acid tolerance response; bioprocessing conditions; residual yoghurt whey.

1. Introduction

Fermentative production of lactic acid has steadily placed within the chemical industry as a cost-effective and environmentally benign path for its large-scale manufacture (John et al., 2009; Wee et al., 2006). However, the next step on this successful market consolidation involves the use of cheap feedstocks for a sustainable bio-production through microbial fermentation. Multiple raw materials such as molasses, starchy or lignocellulosic wastes from agricultural and agro-industrial residues have been thus proposed as carbohydrate rich substrates for lactic acid microbial production (Wee et al., 2006). In fact, residual yoghurt whey has been recently employed as inexpensive source for lactic acid production, reducing therefore the end-of-cycle environmental impact of this food processing waste (Alonso et al., 2010). Unlike fermentable carbohydrate rich materials such as agricultural-based residues, residual yoghurt whey may be converted into lactic acid with no pre-

treatment in a single sugars-to-lactic acid fermentation process carried out by *Lactobacillus casei* (Alonso et al., 2010). However, microbial fermentation systems are strongly reliant on the cellular functionality and viability, which may impair the metabolic ability of the microorganism to produce the desired target metabolite (Hewitt and Nebe-von-Caron, 2001; Lopes da Silva et al., 2009).

The characterization of healthy or nonviable microorganisms is therefore essential to determine the real *health status* of the bioprocess, since changes in the underlying physiology strongly influences not only on the cellular robustness but also on the biological performance, leading consequently to a reduced bioprocess yield (Amanullah et al., 2002; Díaz et al., 2010; Hewitt and Nebe-von-Caron, 2004). Multi-parameter flow cytometry has recently emerged as a powerful tool in industrial biotechnology since it enables to monitor physiological heterogeneity within industrially relevant bioprocesses (Alonso et al., 2012a; Amanullah et al., 2002; Herrero et al., 2006; Quirós et al., 2007). Furthermore, bioprocess monitoring through flow cytometry may provide insights into the right fermentation strategy, enabling a better knowledge of the deleterious bioprocessing conditions on the fermentation system otherwise not detected by conventional culture-dependent or biomass estimation approaches (Hewitt et al., 2001; Lopes da Silva et al., 2009). The use of this technique has been indeed helpful to monitor microbial responses at the population level under hostile conditions such as freezing (Rault et al., 2007), starvation (Lopes da Silva et al., 2005), pH- (Baatout et al., 2007) or bile salt-stress conditions (Ben Amor et al., 2002).

Environmental perturbations or deleterious bioprocessing conditions may undoubtedly induce different patterns of physiological changes, reducing not only the microbial fitness but also biological performance (Alonso et al., 2012a, 2012b). In fact, lactobacilli face several stressing challenges during batch cultivation such as pH oscillation (Rault et al., 2009; Wallenius et al., 2012), which could become a presumed bottleneck during lactic acid bio-production under large-scale conditions. However, the impact of different bioprocessing conditions on the physiological responses of *L. casei* during lactic acid bio-production remains largely unexplored to date.

Within this context, the present study aims to evaluate the physiological responses of *L. casei* during a sugars-to-lactic acid bioprocess using the mixed sugar composition of residual yoghurt whey as inexpensive substrate. Physiological states of *L. casei* were therefore evaluated and monitored through a multi-parameter flow cytometric assessment during uncontrolled- and controlled-pH batch cultivations, with the aim of elucidating the fermentation performance, cellular behaviour and robustness of *L. casei* under acid or non-acid conditions. These results may provide valuable guidelines on the bioprocessing

conditions for enhanced carbohydrate co-fermentation of *L. casei* under changing environmental transitions at an industrial scale.

2. Material and methods

2.1. Microorganism

Lactobacillus casei ATCC 393, obtained from the American Type Culture Collection (Virginia, USA), was maintained frozen (in 40 % [v/v] glycerol at -20ºC). This strain was subsequently subcultured on MRS (de Man Rogosa and Sharpe, Biokar Diagnostic, France) agar plates, incubated for 48 h at 30ºC and then preserved at 4ºC.

2.2. Yoghurt whey preparation

Whey from expired-date yoghurts was obtained by heat treatment (thermal coagulation) in order to remove fat and major proteins. The remaining fat and proteins were subsequently withdrawn from this liquid fraction by centrifugation at 12,000*g* for 10 min at 4ºC. The resulting yoghurt whey was 1-fold diluted with distilled water (1:1) and adjusted to pH 6.5 (by adding NaOH 1 M) prior to sterilization using a tangential microfiltration device equipped with a PVDF membrane-cassette of 0.22 μm pore size (Millipore, Massachusetts, USA).

2.3. Culture conditions and fermentation experiments

After reactivation on MRS under microaerophilic conditions at 30ºC for 16 h, 10% (v/v) of this culture was used to inoculate 90 mL of yoghurt whey. This preculture was incubated at 37ºC for 20 h and placed at 100 rpm in an orbital shaker (Flyer Aerotron, Infors HT, Bottmingen, Switzerland) and then it was used to seed batch fermentations. Controlled-pH fermentation was performed in a 2-L bioreactor (Bioflo 110, New Brunswick Scientific Co. Inc., NJ, USA) with a final working volume of 1 L. pH was strictly controlled at 6.5 by means of computer-controlled peristaltic pumps via automatic addition of 3 M NaOH. Bioreactor cultivation was conducted with an inoculation level of 10% (v/v), an agitation rate of 50 rpm, and at 37ºC.

Uncontrolled-pH batch fermentation was performed with a final working volume of 1 L at 37ºC in an orbital shaker at 100 rpm. In this case, the preculture stage was supplemented with 2.5 g/L of yeast extract in order to promote acidic conditions during final batch cultivation (Alonso et al., 2010). Samples from fermentation broths were withdrawn periodically to determine bacterial growth and physiological status, whilst cell-free supernatants were stored (at -20ºC) until further analysis. Cultivations were carried out in duplicate as independent experiments.

2.4. Staining procedures

Samples from cultures were harvested by centrifugation at 16,000*g* for 5 min. Before staining, cells were washed twice in phosphate-buffered saline (PBS, pH 7.4, sterile and filtered at 0.22 μm), and adjusted to cellular densities corresponding approximately to 4 x 10^5 cells/mL in the same buffer. For viability assessment, 200 μL from this cellular suspension was then added to the different staining solutions previously prepared. Samples were therefore stained with a mixture of two fluorescent dyes, ChemChrome V6 (CV6, Chemunex, France) and Propidium Iodide (PI, Molecular Probes). CV6 is a fluorogenic ester which only diffuses into viable cells and it is hydrolyzed by intracellular esterase enzymes to give fluorescein, a fluorescent product (Parthuisot et al., 2000). Stock solution was prepared by dilution 1:10 in sterile distilled water (0.22 μm filtered) and 8 μL from this solution was added to the cell suspension which was incubated for 15 min in the dark at room temperature. Whilst CV6 stains viable cells which are featured by enzymatic activity, PI is a fluorescent nucleic acid dye which stains damaged and dead cells. PI binds to DNA to form a red fluorescent DNA-complex which enables the assessment of the membrane integrity since this staining dye can only cross the plasmatic membrane if it is permeabilized, corresponding to those cells whose membranes are compromised. The stock solution of PI (1 mg/mL solution in water, Molecular Probes) was both diluted in sterile distilled water and added afterwards to the cell suspension at a final concentration of 5.25 μg/mL. This mixture was incubated for 30 min at the same conditions as CV6 staining. Gates in the flow cytometric dot plots were established according to control samples. Heat-killed cells (treated at 90ºC for 30 min and immediately cooled on ice), early exponentially growing cells and mixtures containing exponential and heat-killed cells (1:1) were therefore analyzed. An unstained sample was additionally used as negative control.

2.5. Multiparameter flow cytometry

Flow cytometry measurements were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with 488- and 633-nm excitation light source from an argon ion laser. Green fluorescence from CV6-stained cells was collected on the FL1 channel (530 nm), whereas PI fluorescence was registered on the FL3 channel (610 nm). Each analysis was performed in duplicate at a low flow rate setting (4,000 events/s). Fluorescent microspheres (Perfect Count, Cytognos, Spain) were used as internal standards in each sample. For every analysis 2,000 microspheres were acquired and performed at a low flow rate setting. Both data acquisition and analysis were carried out using Cytomics RXP software (Beckman Coulter).

2.6. Analytical determinations

Bacterial growth was measured spectrophotometrically as optical density at 660 nm (Shimazdu, UV 1203 model) after centrifugation of culture samples at 16,000*g* for 5 min. Optical density data was converted to cell dry weight (expressed in grams per litre) using the corresponding previously obtained calibration curve.

The lactic acid, lactose, glucose and sucrose content of cell-free culture samples were measured by high performance liquid chromatography as reported previously by Alonso et al. (2011). Samples were quantified according to analytical standards with HPLC grade: lactose, sucrose and lactic acid were purchased from Chem Service (Pennsylvania, USA), whereas glucose was obtained from Merck (Darmstadt, Germany). The liquid chromatographic system used for analysis (Agilent 1200, Agilent Technologies Inc., California, USA) was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., California, USA) coupled to a refractive index detector. Sulphuric acid (0.450 mM, pH 3.1) was employed as the mobile phase at a flow rate of 0.3 mL/min with the column temperature set at 75ºC. Data acquisition and analysis were performed with ChemStation software (Agilent).

2.7. Calculations

The yields of lactic acid on substrate ($Y_{LA/S}$ in g/g) and on biomass ($Y_{X/S}$ in g/g) were calculated by dividing the amount of lactic acid or biomass produced, respectively, by the total amount of sugars consumed. Specific sugar uptake rates (q_S in g/g DCW h) and specific lactic acid production rate (q_{LA} in g/g DCW h) were calculated by linear regression of the concentrations divided by the average biomass concentration at each interval.

3. Results

3.1. Assessment of L. casei physiological status

Figure 1 shows the flow cytometric dot plot disposition of *L. casei* cells, combining CV6 and PI as staining dyes. This dual parameter flow cytometric assessment enabled to distinguish up to three subpopulations: metabolically active, damaged and dead cells (Fig.1). Whereas healthy cells were not stained by PI due to the impermeability of the intact membrane to this nucleic acid dye, damaged cells (double stained cells) exhibited both enzymatic activity and compromised membranes. These sub-lethally injured cells are featured for still maintaining metabolic activity as result of multiple changes in the gene expression pattern which involves either low growth or reduced survival (Díaz et al., 2010; Hewitt and Nebe-von-Caron, 2004). Likewise, healthy cells undergoing physiological stress may be lead to either dead or damaged physiological status since membrane damage involves energy depletion due to the lack of proton motive force (Díaz et al., 2010).

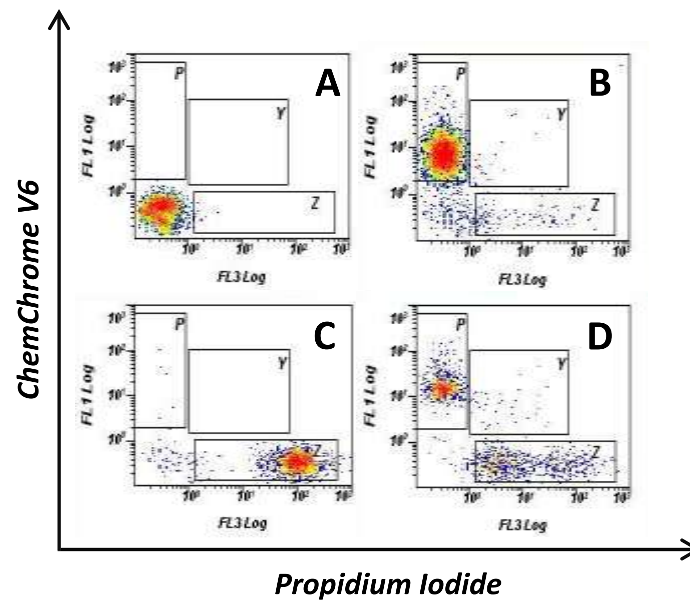

Figure 1. Distribution of *L. casei* cells within a flow cytometric dot plot. Panels show double stained (CV6/PI staining) samples used as flow cytometric controls: unstained cells (A), cells at early-exponential growth phase (B), heat-killed cells (C) and mixture containing early-exponential phase and heat-killed cells (1:1) (D). Gate P in dot plots shows healthy viable cells (CV6(+)/PI(-) cells), while double stained cells (CV6(+)/PI(+) cells) representing sub-lethally damaged *L. casei* cells are shown in gate Y. Cells located in gate Z correspond to dead cells (CV6(-)/PI(+) cells) that exhibited irreversible damages in plasmatic membranes.

3.2. Description of L. casei physiological states during uncontrolled-pH batch cultivation.

As can be seen in Fig. 2, three main subpopulations were clearly distinguishable (metabolically active, damaged and dead cells) during uncontrolled-pH batch cultivation. Bioprocess parameters reveal the existence of carbohydrate co-metabolism by *L. casei* on the mixed carbohydrate composition of residual yoghurt whey, as previously reported (Alonso et al., 2010). Both glucose and sucrose were consumed as the preferential sugars to support *L. casei* growth and metabolism (Fig. 3a). Although batch cultivation was featured by a predominant co-consumption of glucose and sucrose since the beginning, lactose uptake was not lapsed at any time as shown in Fig. 3a. In fact, marked differences in the specific carbohydrate consumption rates were found, with values of 0.5, 0.36 and 0.32 g/gDCW.h for glucose, sucrose and lactose, respectively. This uncontrolled-pH fermentation cultivation was consequently not influenced by the unavailability of preferential carbohydrates since 30.6 g/L of residual sugars were left after 48 h (Fig. 3a).

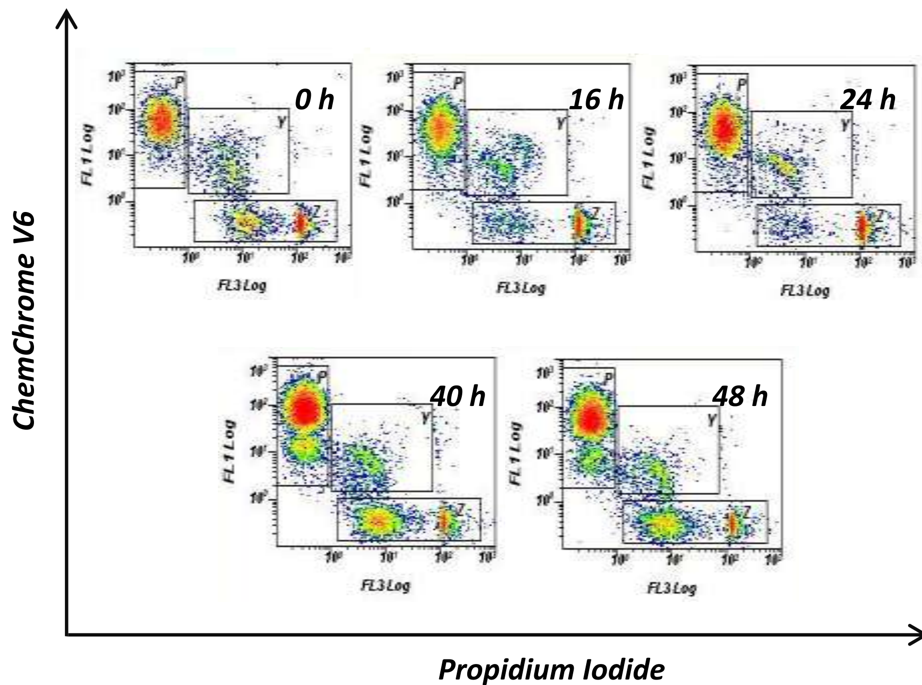

Figure 2. Cytograms representing physiological subpopulations of *L. casei* during uncontrolled-pH fermentation for lactic acid bio-production from yoghurt whey. Cultivation was dominated by three main functional subpopulations: metabolically active, damaged and dead cells.

In terms of lactic acid production, a titer of 18.8 g/L with a yield of 0.67 g/g on sugars and a volumetric productivity of 0.39 g/L.h were achieved after 48 h (Table 1). Obviously, the absence of pH-control caused end-product inhibition and subsequently metabolic feedback repression, leading therefore to a slow hierarchical bioconversion of carbohydrates into lactic acid (Fig. 3a).

As regards physiological heterogeneity, cultivation displayed a robust physiological status, remaining metabolically active most of *L. casei* cells (~70%) throughout cultivation (Fig. 3b). The onset of the stationary growth phase was thus accompanied by the maintenance on viability values notwithstanding of the acidity broth reached (pH<3.6). Both damaged and dead subpopulations remained therefore at values of 8 and 24%, respectively, throughout uncontrolled-pH batch cultivation.

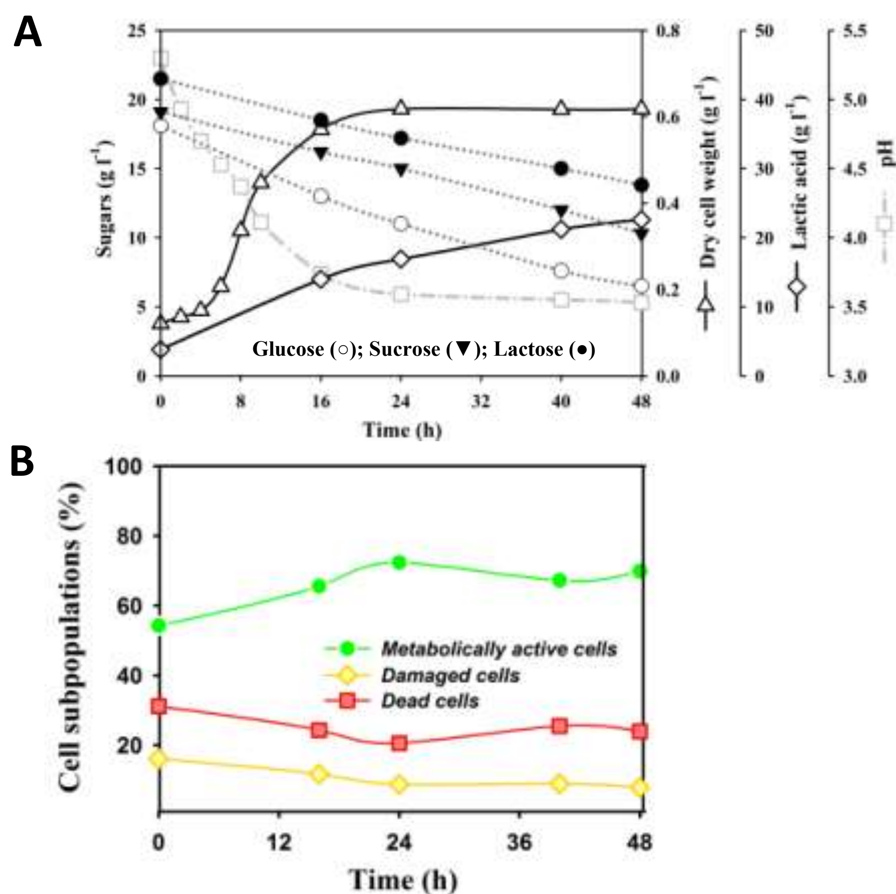

Figure 3. Time-course profile of bioprocess parameters obtained during uncontrolled-pH cultivation (A). Percentages of *L. casei* cell subpopulations throughout the uncontrolled-pH fermentation (B).

Table 1. Comparison of bioprocess parameters obtained in each fermentation process.

	Fermentation process	
	Uncontrolled-pH	***Controlled-pH***
Lactic acid production, P_{LA} (g/L)	18.8	41.5
Yield on substrate, $Y_{LA/S}$ (g/g)	0.67	0.91
Yield on biomass, $Y_{X/S}$ (g/g)	0.02	0.02
Volumetric productivity, Q_{LA} (g/L h)	0.39	0.86
Residual total sugars (g/L)	30.6	13.7
Specific glucose uptake rate, q_{Glc} (g/g DCW h)	0.50	0.79
Specific sucrose uptake rate, q_{Suc} (g/g DCW h)	0.36	0.51
Specific lactose uptake rate, q_{Lac} (g/g DCW h)	0.32	0.40
Specific lactic acid production rate (g/g DCW h)	0.76	1.20

3.3. Controlled-pH batch cultivation: physiological heterogeneity and description of L. casei physiological states.

In most lactobacilli fermentations, lactic acid yield can be ameliorated avoiding end-product inhibition by pH-control cultivation strategies (Schepers et al., 2002; Wallenius et al., 2012). In order to further clarify the role of pH-control strategy on *L. casei* behaviour, batch cultivation was carried out at 6.5. As shown in Fig. 4, cytograms revealed that controlled-pH cultivation suffered from a progressively increase in damaged and dead subpopulations. Conversely, controlled-pH cultivation proceeded at higher lactic acid production and sugar consumption rates than uncontrolled-pH batch culture (Fig. 5a). Lactic acid volumetric productivity was thus remarkably enhanced, resulting in a 1.2-fold (0.86 g/L.h) higher than that achieved under acidic conditions (0.36 g/L.h). Table 1 shows a comparison between bioprocess values obtained under uncontrolled- and controlled-pH conditions.

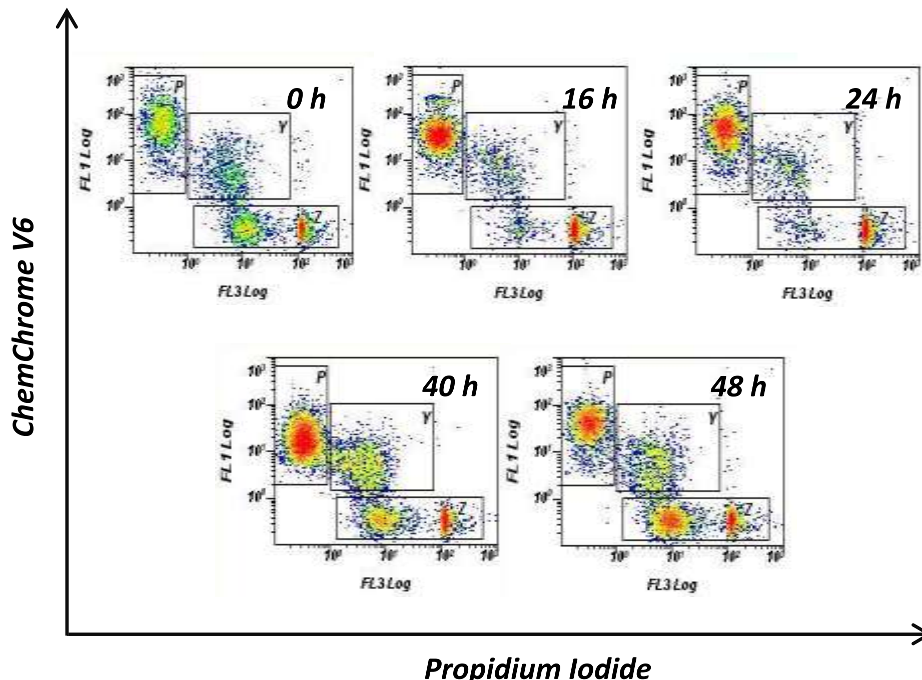

Figure 4. Dot plots representing CV6 fluorescence versus PI fluorescence of *L. casei* cells during controlled-pH batch cultivation at 6.5 on residual yoghurt whey.

L. casei cells also displayed simultaneous carbohydrate co-consumption as Fig. 5a shows, although meaningful higher sugar uptake (0.79, 0.51 and 0.4 g/gDCW.h for glucose, sucrose and lactose, respectively) and lactic production rates (1.2 g/gDCW.h) were attained in comparison to those achieved under uncontrolled-pH conditions (Table 1). Interestingly, the

culture was featured by the depletion in the content of preferential sugars at later stages, leaving only 2.2 and 2.7 g/L of glucose and sucrose after 48 h, respectively (Fig. 5a). As Fig. 5a shows, the pattern in sugar co-utilization was not modified, being glucose and sucrose the preferred carbon sources. Depending on the mixed sugars, microorganisms may exhibit sequential, simultaneous or mixed consumption patterns. In fact, Adler et al. (2012) have demonstrated that the availability of preferred sugars during lactic acid production from lignocellulose may induce changes in the consumption behaviour of *Pediococcus pentosaceus*.

As can be seen in Fig. 5b, *L. casei* displayed a stress-induced physiological response during controlled-pH cultivation. Physiological-state dynamics of *L. casei* showed therefore that dead and damaged subpopulations increased concomitantly to a decline in metabolically active subpopulation. The progressive decrease in cellular viability (from 70 to 40%) after the onset of the stationary growth phase suggests that the amount of lactate produced (41.5 g/L) led to stressful bioprocessing conditions for *L. casei* cells.

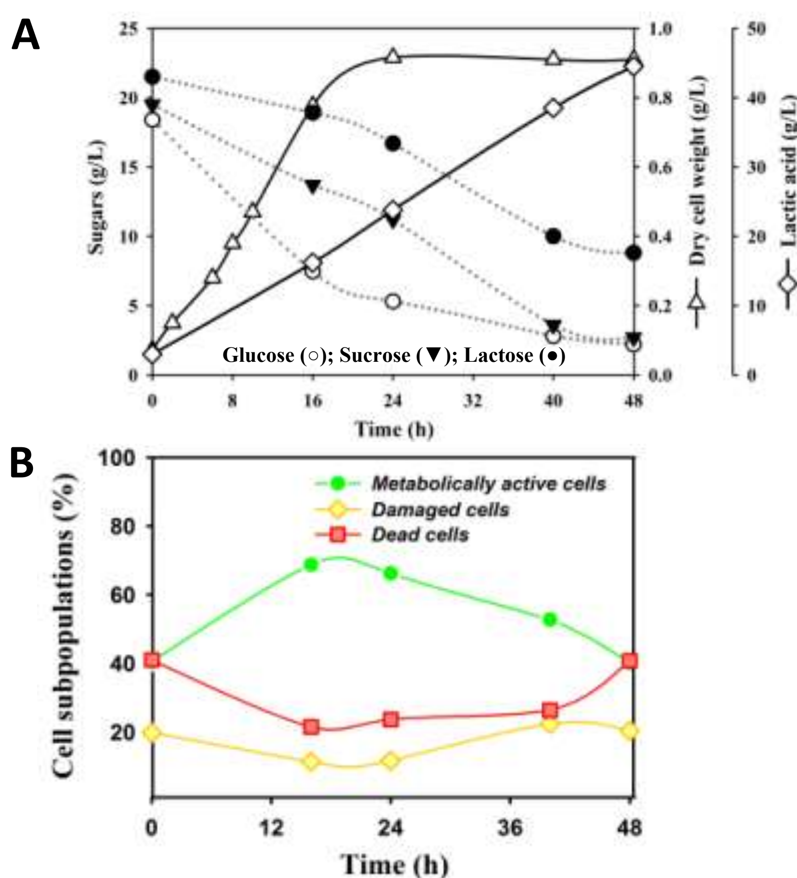

Figure 5. Time-course profile of bioprocess parameters obtained during controlled-pH batch cultivation (A). Percentages of *L. casei* cell subpopulations throughout the controlled-pH fermentation (B).

4. Discussion

While the current lactic acid bio-production market is based on the use of fermentable sugars derived from food crops such as sugarcane and corn, recent efforts have focused on the conversion of inexpensive feedstocks such as lignocellulose and agro-industrial wastes to lactic acid, which can emphasize the cost-effectiveness and sustainability of fermentation systems (Alonso et al., 2010; John et al., 2009; Wee et al., 2006). *L. casei* is a well-known microbial platform for industrial-scale bioproduction of lactic acid not only due to its high metabolic efficiency but also its ability for producing L-lactic acid. However, significant stress physiological responses may hinder biological performance of *L. casei*. In the present study, physiological-state dynamics of *L. casei* during an efficient sugars-to-lactic acid fermentation system was monitored through multiparameter flow cytometry.

Lactobacilli face with multiple and diverse environmental changes during bioprocessing which may trigger relevant physiological responses on a population level. Nutrient starvation, inappropriate pH or osmotic strength may indeed lead to an adaptive physiological response by lactobacilli to these stressing conditions (van de Guchte et al., 2002). Among those bioprocessing challenges, pH may a play a fundamental role in the cellular homeostasis of microorganisms (Nicolau et al., 2010). In fact, stress-induced physiological responses have been found in industrially-relevant fermentation systems due to acid bioprocessing conditions, reducing therefore fermentation performance (Alonso et al., 2012a, 2012b).

Despite the acid environment, *L. casei* displayed a robust physiological status under uncontrolled-pH conditions, suggesting the development of an acid tolerance response (Fig. 3b). These mild acidic conditions (pH<3.7 from 16 h onwards) were not detrimental on the metabolically active subpopulation, which remained at values around 70% during stationary growth phase. Whereas pH stress may result in a loss of cellular functionality and viability in diverse microorganisms (Alonso et al., 2012a, 2012b; Baatout et al., 2007), Lactobacilli are capable of inducing an acid tolerance response in response to mild acid conditions, including pH homeostasis, protection and repair mechanisms (van de Guchte et al., 2002). In contrast, the synergistic negative influence of respiration and pH stress may involve the formation of radicals and loss of membrane integrity in *Lactobacillus plantarum* (Watanabe et al., 2012). Specifically, the acid tolerance response in *L. casei* involves a diverse transcriptional response for enhanced aciduránce (Broadbent et al., 2010). In fact, *L. casei* combats acid stress by maintaining cell membrane functionality through alteration in membrane fluidity and fatty acid distribution (Wu et al., 2012). Besides, the maintenance of

intracellular pH during acidification of the environment requires the modification of cellular metabolism (Rault et al., 2009). Genes related to H^+-ATPase are therefore upregulated in *L. casei* in order to maintain cytoplasmic pH at values close to neutral (Chen et al., 2009). Maintaining the extracellular pH at a high value stabilizes intracellular pH of lactobacilli, decreasing therefore the inhibiting effect of lactic acid (van de Guchte et al., 2002).

Conversely, *L. casei* cells could not withstand stressful bioprocessing conditions imposed by dissociated lactate accumulation (up to 41.5 g/L) under controlled-pH conditions (Fig. 5). These stressful bioprocessing conditions encountered during lactate-producing phase lead therefore to a progressive drop in metabolically active cells due to the damage in membrane integrity. Lactobacilli viability may be particular sensitive to lactate, including an inhibitory growth effect of dissociated form (Yáñez et al., 2008). Rault et al. (2009) have addressed the effect of dissociated lactate level on *Lactobacillus bulgaricus* physiological status. These authors have found that lactate may be important chemical stressor for the lactic acid bacteria during controlled-pH cultivations carried out at pH values of 6. The elevated content of the ionic lactate form at high pH values damaged thus membrane integrity that led to a viability decrease (Rault et al., 2009). Viability of *Lactobacillus helveticus* cultures was indeed better at pH 4.2 than cultivations performed at pH 5.5 (Schepers et al., 2002). Overall, these results suggest that strategies involving continuous cultivation of *L. casei* at high pH control values may result not only in a long-term loss of metabolic efficiency but also in a loss of cellular functionality due to the lack of membrane integrity.

Both fermentative ability and metabolic efficiency of *L. casei* were not altered due to the physiological deleterious bioprocessing conditions encountered during pH-controlled cultivation (Fig. 5a). In terms of lactic acid bio-production, titer was 2.2-fold (41.5 g/L) higher in controlled-pH cultivation than that achieved under acidic conditions (18.8 g/L). Interestingly, sugar consumption profile did not change under these circumstances. The simultaneous co-fermentation of glucose, sucrose and lactose was therefore maintained despite the stress-induced physiological response or acid tolerance response developed by *L. casei* cells under controlled- and uncontrolled-pH conditions, respectively (Figs. 3 and 5). Furthermore, the revealed preferential consumption pattern (glucose>sucrose>lactose) was maintained over the course of the cultivations, suggesting that availability of preferred sugars governed and adjusted the specific carbohydrate palate of *L. casei* regardless the physiological status of the culture.

These results provided a link between the physiological status and lactic acid production by *L. casei*, representing a potential target for cellular robustness efforts designed to produce higher titer of lactic acid from carbohydrate rich sources. Information from

multiparameter flow cytometry assessment provided additionally valuable guidelines on the bioprocessing conditions for enhanced co-fermentation of *L. casei* under changing environmental transitions. Therefore, a lower pH control value may allow the *L. casei* cells not only to maintain a more robust physiological state but also to avoid the end-product inhibition of uncontrolled-pH cultivation strategies. Whereas lactic acid affects pH homeostasis of *L. casei* leading to an inhibition of cellular reactions, lactate concentration may strongly affect membrane integrity and cellular viability.

5. Conclusions

In the present study, results have revealed for the first time how the bioprocessing conditions during an industrially relevant sugars-to-lactic acid bioprocess can lead to cellular stress responses with substantially deleterious effects on the physiological status of *L. casei*. Whereas poor co-fermentation performance was obtained under acidic bioprocessing conditions (obtaining a lactic acid titer of 18.8 g/L with a yield of 0.67 g/g on sugars and a volumetric productivity of 0.39 g/L.h), *L. casei* cells displayed an acid tolerance response featured by a robust physiological status despite the acidic conditions encountered (pH<3.6). Conversely, controlled-pH cultivation was featured by a remarkably drop in the viability associated to the presence of high dissociated lactate titer (41.5 g/L). Addressing therefore the deleterious influence of such bioprocessing conditions constitutes valuable information for improving fermentation performance since this flow cytometry approach may provide guidelines on *L. casei* behaviour otherwise not detected by conventional methodologies.

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4.3. Producción de ácido lactobiónico por Pseudomonas taetrolens

En este trabajo se ha descrito la utilización de suero lácteo para la obtención de ácido lactobiónico por fermentación llevada a cabo por *Pseudomonas taetrolens*. El presente trabajo ha contribuido significativamente en el avance del conocimiento asociado a la producción biotecnológica de ácido lactobiónico puesto que introduce conceptos nuevos, no descritos hasta ese momento. Así, en el mismo se ha definido por primera vez cómo existe una fase de proliferación celular y otra etapa de producción asociada a la formación de ácido lactobiónico por *P. taetrolens* (se trata, por lo tanto, de un compuesto que en su génesis es similar a un metabolito secundario). Igualmente, se ha desarrollado un sistema basado en el control de pH en la fase de producción (*pH-shift strategy*) con el fin de reducir la posible inhibición causada por la acidez generada en un proceso sin control de pH. También se ha enfatizado en los siguientes conceptos como son: el empleo del suero lácteo como materia prima, el incremento del pH asociado a la fase de proliferación celular (salto en el pH o *pH-shift value*), la entrada en fase de producción asociada al incremento en los valores de oxígeno disuelto, el efecto positivo del tamaño de inóculo tanto a nivel de matraz como de biorreactor sobre el rendimiento y la productividad del sistema (variable habitualmente infravalorada en otros sistemas), el desarrollo del proceso sin control de pH a escala de matraz, la influencia del tamaño de inóculo sobre la tasa específica de crecimiento o el desarrollo de una nueva metodología de *screening* oxidativo en placa de agar no descrita hasta la fecha. Todos ellos han sido conceptos que se recogen por primera vez en el artículo científico que a continuación se muestra.

En suma, en este trabajo se ha definido por primera vez un sistema de producción biotecnológica de ácido lactobiónico caracterizado por el empleo de un sustrato con bajo coste. Asimismo, en este trabajo se sentaron las bases para posteriores implementaciones del bioproceso, que servirían para incrementar los valores asociados a la producción biotecnológica.

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Efficient lactobionic acid production from whey by *Pseudomonas taetrolens* under pH-shift conditions

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ABSTRACT

Lactobionic acid finds applications in the fields of pharmaceuticals, cosmetics and medicine. The production of lactobionic acid from whey by *Pseudomonas taetrolens* was studied in shake-flasks and in a bioreactor. Shake-flask experiments showed that lactobionic acid was a non-growth associated product. A two-stage pH-shift bioconversion strategy with a pH-uncontrolled above 6.5 during the growth phase and maintained at 6.5 during cumulative production was adopted in bioreactor batch cultures. An inoculation level of 30% promoted high cell culture densities that triggered lactobionic acid production at a rate of 1.12 g/L h. This methodology displayed efficient bioconversion with cheese whey as an inexpensive substrate for lactobionic acid production.

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1. Introduction

Currently, about 30% of annual world cheese whey production (177 million tonnes in 2006) remains underutilized, ending up as waste or being employed as animal feed (Affertsholt, 2007). Environmental and economic concerns demand that more of the whey should be converted to value-added products. Whey has been explored as a source for producing polyhydroxyalkanoates (Koller et al., 2008; Povolo et al., 2010), ethanol (Guimarães et al., 2010) or hydrogen (Venetsaneas et al., 2009), and the conversion of lactose into lactobionic acid has been proposed as a novel promising solution (Gänzle et al., 2008).

Lactobionic acid displays antioxidant, biodegradable, biocompatible and chelating properties that make it useful in biodegradable detergents (Gerling, 1998), anti-corrosive coatings (Gerling, 1998) as well as sugar-based surfactants (Oskarsson et al., 2007). Lactobionic acid is used in the manufacture of pharmaceuticals, antibiotics and preservative solutions for organ transplants that suppress tissue damage during organ storage (D'Alessandro et al., 1999; Hart et al., 2002). It is also employed as an anti-aging and keratinizing agent in cosmeceutical formulations (Green et al., 2009), and it has attracted attention as part of drug delivery systems (Lin et al., 2009; Wu et al., 2009), biocompatible scaffolds for tissue engineering (Chung et al., 2002) and nanoplatforms for new bioimaging applications (Selim et al., 2007; Kekkonen et al., 2009). Lactobionic acid is used as a preservative, antioxidant and stabilizer-gelling agent (Gerling, 1998) and has been approved as a food additive by the US FDA. This substance is also considered to confer potential prebiotic effects in functional foods (Saarela et al., 2003; Schaafsma, 2008).

At present, lactobionic acid is obtained via chemical synthesis, but this methodology involves harmful catalysts in an energy-intensive process and generates undesirable side-reaction products (Chia et al., 2008; Kuusisto et al., 2007; Murzina et al., 2008). Enzymatic synthesis by oxidation of lactose is possible (Satory et al., 1997; Nordkvist et al., 2007; Van Hecke et al., 2009), but the process is costly and requires redox mediators or cofactor regeneration (Splechtna et al., 2001; Ludwig et al., 2004; Maischberger et al., 2008; Van Hecke et al., 2009).

Whole-cell bioconversion for lactobionic acid production carried out by *Pseudomonas* sp. proceeds slowly and yields lower bioconversion rates (Stodola and Lockwood, 1947) than enzymatic approaches (Pedruzzi et al., 2011), but improvements in yield and costs could be achieved by developing efficient production strategies and utilizing cheap raw materials such as whey. Therefore, in the present study, a pH-shift cultivation strategy was devised to increase bioconversion efficiency by varying inoculum age and size.

2. Methods

2.1. Microorganism and inoculum preparation

2.1.1. Microorganism

Pseudomonas taetrolens LMG 2336, obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium), was

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maintained frozen (in 40% [v/v] glycerol at −20 °C). This strain was subsequently subcultured on Nutrient Broth (NB, containing 1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone and 5 g/L NaCl) agar plates, incubated for 48 h at 30 °C and then preserved at 4 °C.

2.1.2. Inoculum preparation

A loopful of *P. taetrolens* from a fresh Nutrient Broth agar plate was used to inoculate a 500 mL Erlenmeyer flask containing 100 mL of Nutrient Broth medium. This flask was incubated on an orbital shaker (Infors HT, model Flyer Aerotron, Bottmingen, Switzerland) at 250 rpm and 30 °C for 10 h. Actively growing cells from this culture were then employed as inoculum for the production of lactobionic acid in shake flasks and bioreactor seed cultures containing sweet whey, as subsequently reported.

2.2. Sweet whey preparation

Sweet whey (kindly provided by ILAS, Asturias, Spain) was 1-fold diluted with distilled water (1:1) and adjusted to pH 6.5 (by adding NaOH 6 N) prior to sterilization using a tangential microfiltration device equipped with a PVDF membrane-cassette of 0.22 μm pore size (Millipore, Massachusetts, USA).

2.3. Shake-flask bioconversions

Shake-flask bioconversion experiments were conducted in 500 mL Erlenmeyer flasks containing 100 mL of sweet whey and inoculated with 0.035, 0.07, 0.14, or 0.64 g/L of wet biomass from NB precultures harvested by centrifugation at 11,000*g* for 10 min. These cultures were incubated on an orbital shaker (New Brunswick Sci., NJ, USA) at 250 rpm and 30 °C.

Additionally, Erlenmeyer flasks containing 100 mL of sweet whey were supplemented with yeast extract or peptone at concentrations of 0.5, 1, 2, 2.5 and 5 g/L. The flasks were inoculated with 0.14 g/L of wet biomass from NB inoculum cultures and incubated for 48 h.

Samples were aseptically withdrawn periodically to determine bacterial growth and pH. Biomass was removed by centrifugation at 16,000*g* for 5 min, and the cell-free supernatants were stored frozen (−20 °C) until further analysis. Bioconversion experiments were carried out in triplicate as independent trials.

2.4. Batch cultures in a stirred tank bioreactor

Lactobionic production from sweet whey was performed in a 2-L bioreactor (BioFlo 110, New Brunswick Scientific Co., NJ, USA) with a working volume of 1 l. The bioreactor was equipped with a pH meter (Mettler Toledo, Switzerland) and a polarographic dissolved oxygen electrode (InPro 6830, Mettler Toledo, Switzerland) in order to measure and continuously monitor on-line pH and dissolved oxygen tension (DOT) values, respectively. Batch experiments were conducted under the following conditions: temperature was maintained at 30 °C, the agitation rate was set at 350 rpm, employing an aeration rate of 1 v.v.m. through a ring sparger located at the bottom of the bioreactor vessel. Excessive foam formation was prevented by automatic addition of diluted (1:10) Y-30 emulsion (Sigma–Aldrich, Steinheim, Germany). A two-stage pH-shifted bioconversion strategy was adopted in bioreactor experiments based on preliminary shake-flask results. pH control was activated above 6.5 (pH was left uncontrolled above this value during the growth phase and maintained at 6.5 thereafter) by computer-controlled peristaltic pumps via automatic addition of 2 M NaOH.

The seed culture was obtained by harvesting biomass (after centrifugation at 11,000*g* for 10 min) from a NB shake-flask culture and re-suspending cells in a 500 mL Erlenmeyer flask containing 100 mL of sweet whey. Subsequently, cells were grown in cheese whey at 250 rpm and 30 °C in an orbital shaker for 12, 24 and 36 h. Each culture was employed as seed culture in the bioreactor at 10% (v/v) containing sweet whey to study the effect of inoculum age on culture parameters. The influence of inoculation level on lactobionic acid production was studied with inoculation levels of 5%, 10%, 20% or 30% (v/v) of seed cultures cultivated for 12 h in sweet whey at 250 rpm and 30 °C. Cultivations were carried out in duplicate as independent experiments.

2.5. Analytical methods

Bacterial growth was measured spectrophotometrically as optical density at the 600 nm (Shimazdu, UV 1203 model) after centrifugation of culture samples at 16,000*g* for 5 min. Optical density data was converted to cell dry weight (expressed in grams per liter) using the corresponding calibration curve previously obtained.

Lactobionic acid and lactose contents of culture samples were measured by high performance liquid chromatography following a modified version of a previously published method (Pedruzzi et al., 2007). Analyses were carried out using a liquid chromatography system (Agilent 1200, Agilent Technologies Inc., California, USA) equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., California, USA) coupled to a refractive index detector. As the mobile phase, 0.450 mM H_2SO_4 (pH 3.1) was employed at a flow rate of 0.3 mL/min with the column temperature set at 75 °C. Samples were quantified according to HPLC-grade external analytical standards, lactose was obtained from Chem Service (Pennsylvania, USA), whereas lactobionic acid was purchased from Sigma–Aldrich (Steinheim, Germany). Data acquisition and analysis were performed with ChemStation software (Agilent).

2.6. Lactose-oxidizing capacity of P. taetrolens

A loopful of *P. taetrolens* cells was streaked onto agar plates containing 10 g/L lactose, 2 g/L peptone, 1 g/L yeast extract, 0.2 g/L K_2HPO_4, 0.08 g/L bromophenol blue and 15 g/L agar as an screening medium for oxidation and acid production and incubated at 30 °C for 96 h. The same medium composition, though excluding lactose, was employed as a negative control of lactose-oxidizing capacity.

3. Results and discussion

3.1. Preliminary screening of oxidative bioconversion by P. taetrolens on agar plates

The development of a preliminary plate assay has enabled the screening of the oxidizing capacity of *P. taetrolens* as the presence of bromophenol blue allowed the appearance of clear zones surrounding the lactobionic-producing colonies. Supplementary Fig. 1A and C illustrate color changes in the agar plates. As a result, an observable yellow coloring was formed due to lactobionic acid production and subsequent decrease in pH, verifying the capacity of *P. taetrolens* cells to oxidize lactose after 48 h incubation compared to control plates without the presence of lactose (Supplementary Fig. 1B). This approach has enabled the distinction between the cell growth phase during the first 24 h and the subsequent lactobionic acid production phase on agar plates (Supplementary Fig. 1A). These results also give support to the idea that *P. taetrolens* may oxidize lactose as originally reported for many *Pseudomonas* species by Stodola and Lockwood (1947).

The bioconversion features a single biotransformation to lactobionic acid catalyzed by a membrane-bound lactose oxidation system that catalyzes lactose oxidation to a lactone intermediate (lactobiono-δ-lactone) whose carbonyl group is subsequently

hydrolyzed by a lactonase into lactobionic acid (Fig. 1) (Nishizuka and Hayaishi, 1962).

3.2. Shake-flask cultivations: effect of inoculum size

The influence of inoculum size on lactobionic acid production from cheese whey is shown in Fig. 2. Lactobionic acid production followed a biphasic pattern with an initial phase of cell growth and a second production phase characterized by concomitant broth acidification. Increased inoculum size triggered lactobionic acid production by achieving higher cell culture densities of 1.18, 1.24, 1.36 and 1.86 g/L for 0.035, 0.07, 0.14 and 0.64 g/L of initial biomass, respectively (Fig. 2A). The residual lactose contents also showed a clear influence of inoculum size on lactobionic acid production from whey (Fig. 2C). Employing an initial inoculum of 0.64 g/L of biomass, complete lactose bioconversion was achieved after 60 h versus a yield (defined as the percentage of lactose converted into lactobionic acid after 80 h) of 81% obtained with 0.035 g/L after 80 h (Fig. 2D).

The pH of the cultures remained within a pH range of 6.5–8 until the onset of the stationary growth phase, sharply declining to acidic pH values thereafter as a consequence of lactobionic acid production (Fig. 2B). The pH became slightly basic during the growth phase owing to the appearance of nitrogen by-products from metabolized proteins which raised the broth pH from 6.5 to 7.36 employing 0.64 g/L of biomass as inoculum. Thus, for the cultures with a lower initial biomass, the pH-peaked values during the growth phase were always higher than for those obtained with high initial biomass sizes. Clearly, the influence of initial higher cell densities may have contributed to the improved yield performance in shake-flasks (Fig. 2C and D). The use of greater inoculum sizes progressively shortened the onset of the production phase coinciding with pH-shift. Thus, with an inoculum of 0.64 g/L, the time before the onset of the production phase was 6 h, compared to 16 h for a biomass size of 0.035 g/L (Fig. 2D). Moreover, volumetric productivity was also clearly affected by inoculum size, achieving values of 0.43, 0.45, 0.48 and 0.6 g/L h for 0.035, 0.07, 0.14 and 0.64 g/L of biomass, respectively.

In contrast, the specific growth rate decreased with increasing inoculum sizes as a consequence of limiting substrate concentrations (Fig. 3A). In terms of bioprocess yield, the use of a higher initial biomass concentration had a particularly positive effect on bioconversion efficiency, reaching a maximum above 0.37 g/L of initial biomass (Fig. 3A).

Lactobionic acid may thus be described as a non-growth associated product whose whole-cell oxidative bioconversion from the lactose present in cheese whey was closely related to the cell density achieved after the growth phase. Therefore, shorter growth phases, a higher yield and a significant reduction in bioprocess duration were obtained under high cell-density conditions.

3.3. Effects of nitrogen source supplementation on cell growth and lactobionic acid production at shake-flask scale

The presence of extra nitrogen sources prolonged the growth phase and higher cell densities were obtained in the stationary growth phase, as shown in Tables 1 and 2. As can be seen from Fig. 3B, nitrogen source supplementation with either yeast extract or peptone increased the specific growth rate and it reached a maximum when using 5 g/L of nitrogen supplementation of 0.25 and 0.26 h^{-1} for yeast extract and peptone supplementation, respectively.

In terms of yield (defined as the percentage of lactose converted into lactobionic acid after 48 h), values varied from 74% to 88% and from 72% to 84% for yeast extract and peptone supplementation experiments, respectively, suggesting improved bioconversion performance in the presence of yeast extract, although the delay in the production phase was slightly higher than for the peptone experiments (Tables 1 and 2). However, these high cell density conditions after 48 h did not lead to higher yields in comparison to those obtained without extra nitrogen sources (87%), indicating that *P. taetrolens* cells were induced towards greater cell proliferation rather than product formation in the presence of extra nutrients. Lactobionic acid production might be affected by the oxygen-limited culture conditions encountered by *P. taetrolens* cells during a prolonged cell proliferation phase.

3.4. Effect of seed culture age on lactobionic acid production

As Fig. 4 shows, the lag phases increased significantly with the use of cells from inocula in the stationary growth phase. A lag phase of 4 and 8 h was respectively detected in bioreactor cultivations at 24- and 36-h, whereas an absence of the lag phase was observed when employing a 12-h seed culture (Fig. 4). The maximum dry cell weight varied as a function of inoculum age and was higher for the youngest inoculum (1.25 versus 1.01, 0.98 g/L of biomass being obtained for cultivations seeded with 24 and 36-h cultures, respectively). Due to the lower cell densities obtained in the 24 and 36-h cultures, yields of 91% and 66%, respectively were obtained and the residual lactose concentrations were 3.9 and 14.1 g/L after 82 h for cultivations seeded with 24- and 36-h cultures, respectively. Dissolved oxygen tension (DOT) and pH values remained unchanged during the lag phase, as Fig. 4B and C show. Physiological ages at 24 and 36 h exerted major effects on lactobionic acid production from whey seeing as the length of the lag phase preceding growth increased in comparison to batch cultivation with a 12-h seed culture.

Overall, it may be concluded from these results that the use of time-prolonged seed cultures of *P. taetrolens* led to an increase in the lag phase, as well as a reduction in lactobionic acid yield in cultivations compared to late exponential growth phase inocula. Therefore, a 12-h seed culture in whey was selected as the optimal inoculum for the experiments investigating inoculation level.

3.5. Effect of inoculation level on lactobionic acid production at bioreactor scale

As can be seen in Fig. 5, high inoculum density favored the rapid activation of *P. taetrolens* cells, affecting the onset of the production phase. Thus, with an inoculation level of 30% (v/v), the culture reached the maximum cell density of 1.35 g/L in 4 h with a specific growth rate of 0.15 h^{-1}. Figs. 4A and 5 show the time plots of bio-

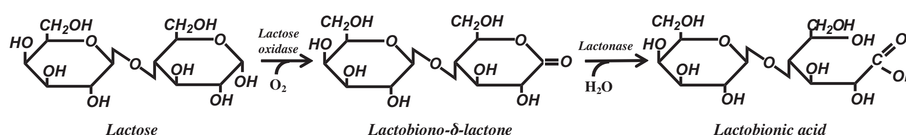

Fig. 1. Bioconversion of lactose to lactobionic acid by *P. taetrolens* is carried out through a membrane-located dehydrogenase system which mediates lactose oxidation and subsequent hydrolysis by lactonase to yield lactobionic acid.

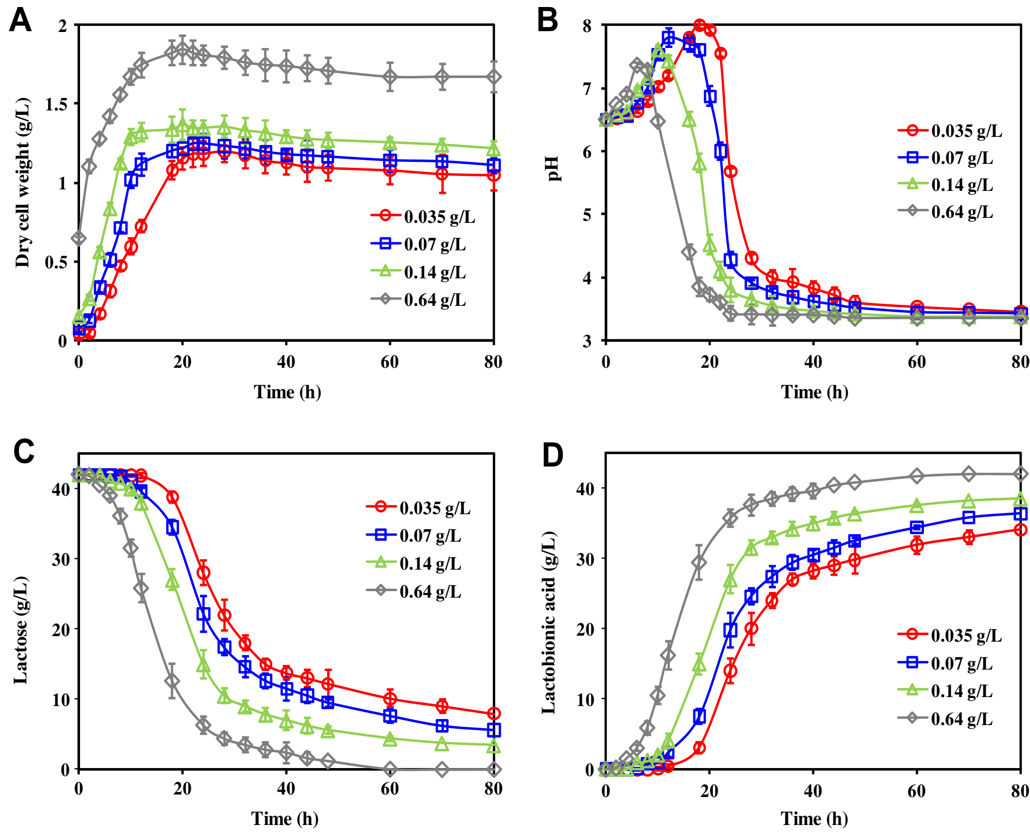

Fig. 2. Effect of inoculum size on biomass (A), pH (B), lactose (C) and lactobionic acid production (D) profiles from whey by *P. taetrolens* in shake-flask cultures. Four initial biomass concentrations (0.035, 0.07, 0.14 and 0.64 g/L) were employed as inoculum in the experiments.

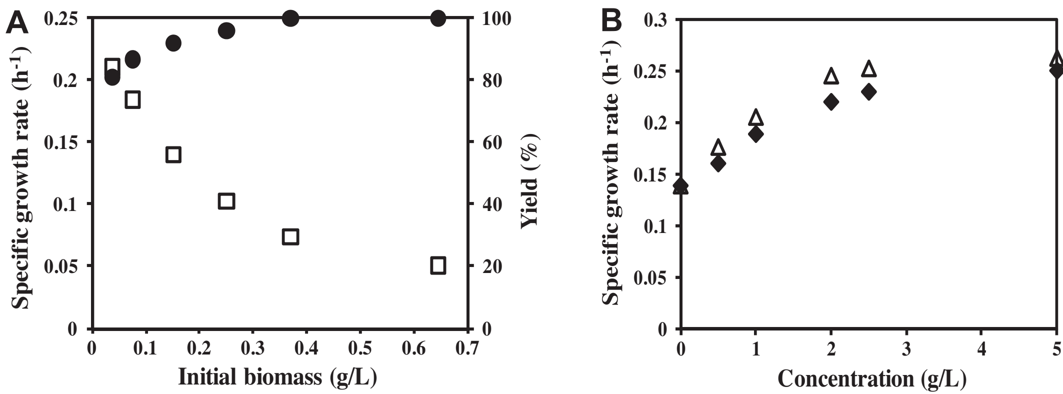

Fig. 3. Influence of initial biomass concentration on the specific growth rate (□) and *P. taetrolens* bioconversion efficiency (●), expressed as percentage of lactose converted into lactobionic acid after 80 h, in sweet whey at shake-flask scale (A). Relationship between specific growth rate and yeast extract (♦) or peptone (△) supplementation on whey for lactobionic acid production (B).

process parameters for a 5%, 10%, 20% and 30% (v/v) inoculation level, corresponding to an initial biomass of 0.09, 0.19, 0.36 and 0.56 g/L, respectively. Table 3 shows the overall bioconversion parameters achieved with the four different inoculation levels employed.

The use of higher inoculation levels also shortened the duration of the growth phase from 10 to 4 h for a 5% and 30% inoculation level, respectively. Moreover, a similar trend to the specific growth rates in shake-flask cultivations was found and the highest specific growth rate was observed for the lowest inoculation level, 5% (0.28 h^{-1}). A significant reduction in bioconversion duration was obtained from 72 to 32 h with a 5% and 30% inoculation level, respectively. Maximum volumetric lactobionic acid productivity of 1.12 g/L h was thus achieved with the highest inoculation level compared to 0.59 g/L h using the lowest inoculation level (Table 3). This maximum volumetric productivity obtained was thus 2.4-fold higher than that attained by Miyamoto et al. (2000) who reported around 0.46 g/L h in batch mode after 96 h. Undoubtedly, whole-cell bioconversions often proceed with lower efficiencies and longer operation times compared to those achieved with biocatalytic/enzymatic approaches like the one employed by Pedruzzi et al. (2011), which can yield lactobionic acid titers of around 125.4 g/L from 252 g/L of chemically pure lactose solution.

The pH-shift values correlated with inoculation levels (Table 3) and increased levels led to a decrease in pH-shift values. The pH increased to about 8.13 (at 11 h), 8.00 (at 9 h), 7.60 (at 6 h) and 7.30

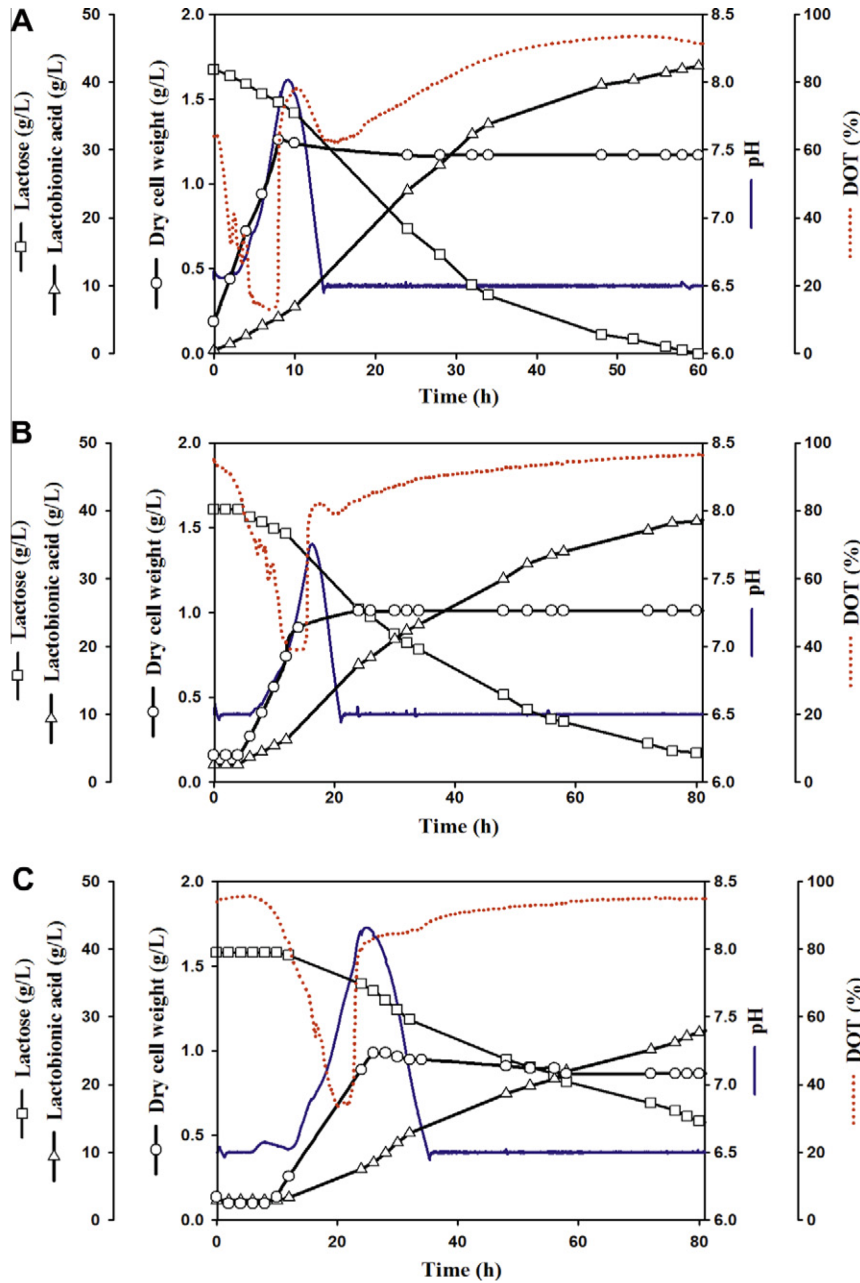

Fig. 4. Effect of seed age on lactobionic acid production from whey by *P. taetrolens*. Time-course profiles of bioprocess parameters during batch bioconversion in a bioreactor employing 12-h (A), 24-h (B) and 36-h (C) seed cultures.

Table 1
Effect of yeast extract supplementation on growth and lactobionic acid production from whey by *P. taetrolens* after 48 h.

YE concentration (g/L)	Maximum DCW obtained (g/L)	μ (h^{-1})	Onset of production phase (h)	Yield (%)
0.5	1.36 ± 0.08	0.16	12	74
1	1.38 ± 0.06	0.19	12	76
2	1.54 ± 0.06	0.22	14	79
2.5	1.78 ± 0.12	0.23	15	81
5	2.24 ± 0.03	0.25	16	88

YE = yeast extract; DCW = dry cell weight; μ = specific growth rate.

Table 2
Effect of different concentrations of peptone on growth and lactobionic acid production from whey by *P. taetrolens* after 48 h.

Peptone concentration (g/L)	Maximum DCW obtained (g/L)	μ (h^{-1})	Onset of production phase (h)	Yield (%)
0.5	1.35 ± 0.07	0.18	12	72
1	1.36 ± 0.05	0.20	12	74
2	1.48 ± 0.03	0.24	14	77
2.5	1.75 ± 0.1	0.25	14	78
5	1.97 ± 0.02	0.26	15	84

DCW = dry cell weight; μ = specific growth rate.

(at 5 h) for a 5%, 10%, 20%, 30% inoculation level, respectively, subsequently decreasing to 6.5 during the production phase (Figs. 4A and 5). The batch bioconversion seeded with a 5% (v/v) of inoculum volume showed the highest culture broth alkalinity (pH of 8.13)

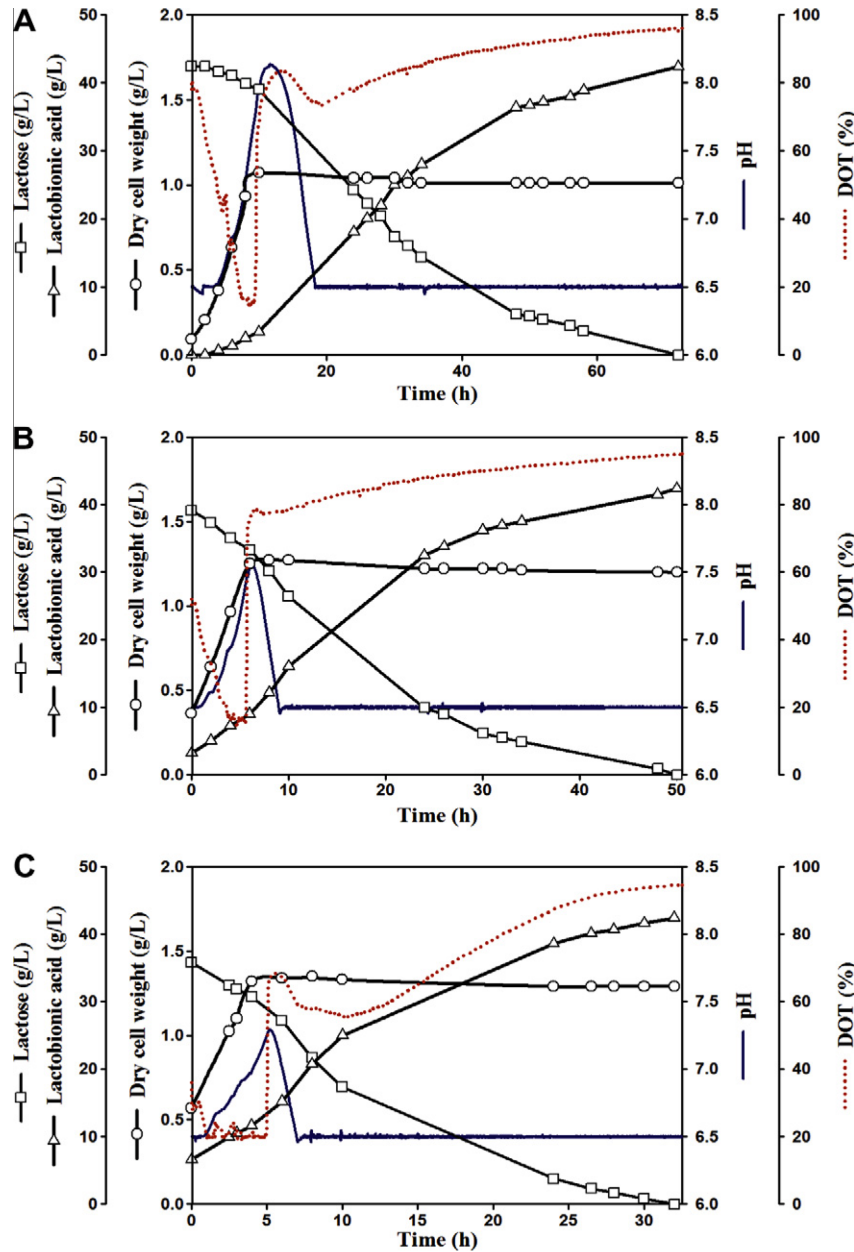

Fig. 5. Time profiles of biomass, pH, DOT, lactose and lactobionic acid during pH-shift bioreactor cultivations with a 5 (A), 20 (B) and 30% (v/v) (C) inoculation level.

Table 3
Summary of values obtained under the influence of inoculation level on batch bioconversion at bioreactor scale.

Inoculation level% (v/v)	Maximum dry cell weight (g/L)	Duration of growth phase (h)	pH-shift value	μ (h^{-1})	Volumetric productivity (g/L h)	Time (h)	Yield (%)
5	1.08	10	8.13	0.28	0.59	72	100
10	1.25	8	8.00	0.19	0.69	60	100
20	1.27	6	7.60	0.17	0.80	50	100
30	1.35	4	7.30	0.15	1.12	32	100

(Fig. 5A), whereas the maximum pH value obtained in the bioreactor when employing a 30% inoculation level was 7.30 (Fig. 5C).

During the growth phase, a sharp drop in dissolved oxygen tension values was found, but a sharp rise in the saturated dissolved oxygen concentration occurred after nutrient depletion, signaling the switch from growth to product formation (Fig. 5). High microbial cell densities impacted on oxygen availability during the growth phase period (Fig. 5). Therefore, in order to avoid oxygen-limited cultivation conditions with an inoculation level of 30% (v/v), the DOT was maintained above 20% (Fig. 5C) by automatic control of the agitation speed (up to 500 rpm).

In the bioreactor experiments, lactobionic acid was a non-growth associated product within a bioprocess which proceeded with a concomitant drop in dissolved oxygen concentration as well as an increase in pH values during growth phase.

The elucidation of a suitable and feasible downstream processing methodology after the bioconversion process could guarantee the successful implementation of this system. Considering that the recovery process depends primarily on the nature of the matrix employed for lactobionic acid production, media components and metabolites from bioconversion broth could become a drawback to purify the desired target compared to the lesser effort required

for downstream processing of lactobionic acid produced after catalytic approaches based on refined lactose. After cell harvesting, recovery of lactobionic acid from bioconversion broth could be accomplished by conventional methods involving precipitation, extraction and adsorption using ion-exchange resins. Undoubtedly, lactobionic acid recovery after production processes will be subject of intensive future research.

4. Conclusions

Cheese whey was employed as a raw material for production of lactobionic acid by *P. taetrolens* using a two-stage pH-shifted bioconversion strategy, and lactobionic acid production of 42.4 g/L with a 30% volume seed culture inoculum was obtained after 32 h. Whey offers an alternative means to costly synthetic media with nutrient-mineral supplementation for lactobionic acid production. Further improvements concerning the influence of physical culture parameters on microbial behavior as well as downstream processing are required for implementation of the process on an industrial level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.07.089.

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Appendix A. Supplementary data

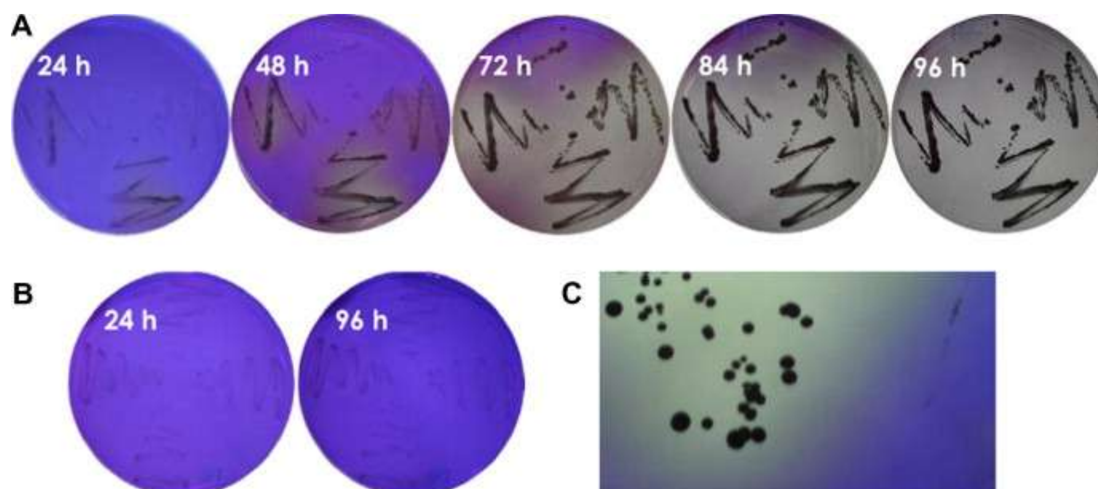

Figure 1 Supplementary data. Details of oxidative bioconversion carried out by *Pseudomonas taetrolens* on agar plates containing lactose. Progressive temporal color changes in an agar plate incubated for 96 h (A). Negative control plates without lactose after 24 and 96 h (B). Detail of yellow clearance zone formed around *P. taetrolens* colonies (C).

4.4. Influencia de la disponibilidad de oxígeno disuelto sobre la producción de ácido lactobiónico

La disponibilidad de oxígeno disuelto en el sistema fermentativo anteriormente desarrollado debería en principio jugar un papel relevante al tratarse de un proceso aeróbico. Por lo tanto, en este subapartado se ha evaluado la influencia de este parámetro sobre el proceso de producción biotecnológica de ácido lactobiónico por *P. taetrolens* a partir de suero lácteo. Es evidente que la producción de este ácido podría verse afectada en el proceso de aireación y/o mezcla como consecuencia de la limitación en el aporte de oxígeno, parámetro que ha de ser tenido en cuenta en todo sistema aeróbico. No obstante, el presente sistema no sólo se caracteriza por la dependencia del oxígeno durante la proliferación y mantenimiento celular (siendo utilizado el O_2 como aceptor final de electrones en la cadena respiratoria) sino que el O_2 es un elemento clave en la oxidación de la lactosa por parte de *P. taetrolens* para dar lugar al ácido lactobiónico ya que re-oxida al cofactor rédox (dinucleótido de flavina-adenina, FAD) involucrado en la formación de dicho ácido (véase el subapartado 2.3 para mayores detalles sobre la reacción). Se han evaluado igualmente la transferencia de oxígeno y el consumo por parte de *P. taetrolens*. De esta forma, se ha comprobado cómo una supresión del aporte de oxígeno puede conllevar un irremediable efecto negativo sobre la producción de ácido lactobiónico. Asimismo, se ha evaluado tanto el impacto de diferentes niveles de aireación (0-2 Lpm) como de agitación (150-1000 rpm) sobre los diferentes valores asociados al sistema (proliferación celular, tasa específica de crecimiento, valor de pH, etc…).

Se ha evaluado también el comportamiento del sistema ante diferentes niveles de oxígeno disuelto en la fase de proliferación celular en condiciones de alta densidad celular inicial (tamaño de inóculo del 30%, v/v). Para ello, se han variado los niveles de oxígeno disuelto en el medio a través de una cascada de agitación, evaluando por lo tanto el comportamiento del sistema bien sea bajo condiciones en exceso o limitadas de aporte de oxígeno.

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Role of dissolved oxygen availability on lactobionic acid production from whey by *Pseudomonas taetrolens*

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ABSTRACT

The influence of dissolved oxygen availability on cell growth and lactobionic acid production from whey by *Pseudomonas taetrolens* has been investigated for the first time. Results from pH-shift bioreactor cultivations have shown that high agitation rate schemes stimulated cell growth, increased pH-shift values and the oxygen uptake rate by cells, whereas lactobionic acid production was negatively affected. Conversely, higher aeration rates than 1.5 Lpm neither stimulated cell growth nor lactobionic acid production (22% lower for an aeration rate of 2 Lpm). Overall insights into bioprocess performance enabled the implementation of 350 rpm as the optimal agitation strategy during cultivation, which increased lactobionic productivity 1.2-fold (0.58–0.7 g/L h) compared to that achieved at 1000 rpm. Oxygen supply has been shown to be a key bioprocess parameter for enhanced overall efficiency of the system, representing essential information for the implementation of lactobionic acid production at a large scale.

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1. Introduction

Lactobionic acid has recently burst onto the cosmetic and pharmaceutical scene as a relevant polyhydroxy acid covering a broad spectrum of applications. In addition to its cosmeceutical use (Green et al., 2009), lactobionic acid is being increasingly employed as a potential drug carrier (Wu et al., 2009; Zhang et al., 2011), as well as in the development of carbohydrate-functionalized nanoparticles used as therapeutic agents for biomedical purposes (Lin et al., 2009). Considering that its commercial relevance is undergoing stepwise growth (Affertsholt, 2007), both the development and implementation of feasible lactobionic production systems appear as crucial key challenges to meet market demands.

Presently, emerging considerations regarding environmental regulatory frameworks are moving the dairy industry towards more sustainable practices. As a result, cheese whey has been the subject of valorization either to obtain different value-added bioproducts (Koutinas et al., 2009; Guimarães et al., 2010) or to reduce its pollution potential (Ghaly and Kamal, 2004).

Within this context, cheese whey has been proved to be a suitable, inexpensive and attractive source for obtaining lactobionic acid through a biotechnological process carried out by *Pseudomonas taetrolens* (Alonso et al., 2011). However, as submerged microbial bioprocesses involving aerobic platforms are often strongly influenced by mass transfer limitations, the availability of dissolved oxygen to microbial cells must accordingly be considered (García-Ochoa and Gómez, 2009). Both monitoring and proper control of dissolved oxygen levels represent decisive factors in aerobic systems seeing as insufficient oxygen supply may inhibit metabolism, cell growth and microbial biosynthesis. Owing to their intrinsic nature, oxygen as well as agitation regimes have been shown to play a major role in numerous submerged liquid systems involved in gellam (Giavasis et al., 2006), hyaluronic acid (Huang et al., 2006), ganoderic acid (Tang and Zhong, 2003), alginate (Peña et al., 2000), enzyme (Rahulan et al., 2011) or xantham gum synthesis (Amanullah et al., 1998). Consequently, the elucidation of suitable aeration supply schemes enables the generation of a suitable microenvironment for improved cellular proliferation patterns, as well as higher metabolite production yields.

In industrial practice, the mass transfer coefficient (K_La) as well as oxygen requirements by microbial cells (measured as the oxygen uptake rate, OUR) are used as scale-up criteria (Zou et al., 2008; García-Ochoa and Gómez, 2009) given that K_La is essentially a measure of how much oxygen can be supplied to microorganisms growing in a bioreactor (Bandyopadhyay et al., 2009). Specifically, oxygen mass transfer has been chosen as the controlling step rate for the overall behavior in oxygen-limited fermentation processes (García-Ochoa et al., 2000; Lozano et al., 2011). In fact, a concise understanding of the key oxygen parameters associated with any submerged liquid culture is crucial to enhance microbial performance (Wang et al., 2010; Xu and Zhong, 2011).

The aim of the present study was thus to investigate the relationship between dissolved oxygen and biotechnological production of lactobionic acid from whey by *P. taetrolens*, exploring for

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the first time bioconversion performance under oxygen-limited and non-oxygen-limited conditions. To this end, bioreactor cultivations were performed under different aeration and agitation rates in order to examine whether such effects would have an impact on lactobionic acid synthesis. Two bioprocess parameters were also evaluated, namely the oxygen uptake rate and the volumetric oxygen transfer coefficient, thereby enabling improved knowledge of the role of oxygen in culture performance and providing insights into the most suitable oxygen supply scheme for optimal lactobionic acid production from whey.

2. Methods

2.1. Microorganism

Pseudomonas taetrolens LMG 2336, obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium), was maintained frozen (in 40% [v/v] glycerol at −20 °C). This strain was subsequently subcultured on Nutrient Broth (NB, containing 1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone and 5 g/L NaCl) agar plates, incubated for 48 h at 30 °C and then preserved at 4 °C.

2.2. Inoculum and seed culture preparation

A loopful of *P. taetrolens* from a fresh Nutrient Broth agar plate was used to inoculate a 500 mL Erlenmeyer flask containing 100 mL of NB medium. This flask was incubated on an orbital shaker (Infors HT, model Flyer Aerotron, Switzerland) at 250 rpm and 30 °C for 10 h. Actively growing cells from this culture were then harvested by centrifugation at 11,000×g for 10 min and re-suspended in a 500 mL Erlenmeyer flask containing 100 mL of whey. This flask was incubated at 250 rpm in an orbital shaker at 30 °C for 12 h and subsequently employed as seed culture in the bioreactor experiments containing sweet whey.

2.3. Sweet whey preparation

Cheese sweet whey (provided by ILAS, Asturias, Spain) was 1-fold diluted with distilled water (1:1) and adjusted to pH 6.5 (by adding NaOH 6 N) prior to sterilization using a tangential microfiltration device equipped with a PVDF membrane-cassette of 0.22 μm pore size (Millipore, Massachusetts, USA).

2.4. Batch cultivations in stirred tank bioreactor

Batch cultivations were performed in a 2-L bioreactor (BioFlo 110, New Brunswick Scientific Co., New Jersey, USA) with 1 L of whey as working volume. Bioreactor experiments with an inoculation level of 10% (v/v) were conducted at 30 °C. The bioreactor was equipped with a pH meter (Mettler Toledo, Switzerland) and a polarographic dissolved oxygen electrode (InPro 6830, Mettler Toledo, Switzerland) in order to measure pH values on line and continuously monitor dissolved oxygen tension (DOT), respectively. Excessive foam formation was prevented by automatic addition of diluted (1:10) Y-30 emulsion (Sigma–Aldrich, Steinheim, Germany). An efficient two-stage pH-shifted bioconversion strategy was adopted as previously described (Alonso et al., 2011): pH was controlled above 6.5 (pH was left uncontrolled above this value during the growth phase and subsequently maintained at 6.5) by means of computer-controlled peristaltic pumps via automatic addition of 2 M NaOH. These prior conditions were applied to all cultivations unless otherwise specified. Cultivations were carried out in duplicate as independent experiments.

2.5. Influence of oxygen deprivation after the growth phase

Process performance was analyzed by combining a two-stage batch cultivation carried out at a medium agitation rate (350 rpm): an initial aerobic growth phase (1 Lpm during 0–10 h) followed by an oxygen supply deprivation stage after the exponential growth phase (10–60 h).

2.6. Effect of agitation rate on biomass and lactobionic acid production from whey

In order to examine the influence of the agitation rate on bioprocess parameters, bioreactor cultivations were carried out at 150, 350, 500, 700 and 1000 rpm with the aeration rate kept at 1 Lpm.

2.7. Effect of aeration rate on bioprocess parameters

The effect of different aeration rates (0, 0.5, 1, 1.5 and 2 Lpm) on bioprocess parameters was studied in batch cultivations carried out at 350 rpm.

2.8. Effect of dissolved oxygen concentration under high-cell density conditions

In order to gain insight into how the system and bioprocess parameters are affected by the availability of different dissolved oxygen concentration, high-cell density cultivations were performed under several DOT control levels during the exponential growth phase. The DOT level was left free or controlled at 20%, 40% and 60% (percentage of air saturation) by cascading the agitation speed (from 350 up to 500 rpm). For the cell density conditions, cultivations were conducted with an inoculation level of 30% (v/v) at 1 Lpm as aeration rate.

2.9. Determination of the oxygen uptake rate (OUR) and volumetric oxygen transfer coefficient (K_La)

The oxygen uptake rate (OUR) and volumetric oxygen transfer coefficient (K_La) were determined via the dynamic method (Bandyopadhyay et al., 2009; García-Ochoa and Gómez, 2009). The concentration of dissolved oxygen under steady state conditions can thus be formulated as Eq. (1):

$$\frac{dC}{dt} = K_La(C^* - C_L) - q_{O_2}X = 0 \tag{1}$$

where q_{O_2} is the specific oxygen consumption rate, X is the concentration of biomass, while the first term corresponds to the mass transfer rate. Accordingly, the OUR was obtained from the slope of the plot of dissolved oxygen concentration (C_L) versus time following a momentary interruption of air supply to the bioreactor. After aeration was re-established, K_La was calculated from the slope of Eq. (2) by plotting C_L versus ($q_{O_2}X + dC_L/dt$):

$$C_L = C^* - \frac{1}{K_La}\left(q_{O_2}X + \frac{dC_L}{dt}\right) \tag{2}$$

2.10. Analytical methods

Bacterial growth was measured spectrophotometrically as optical density at 600 nm (Shimazdu, UV 1203 model) after centrifugation of culture samples at 16,000×g for 5 min. Optical density data were converted to cell dry weight (expressed in grams per liter) using the corresponding calibration curve obtained previously.

The lactobionic acid and lactose content of cell-free culture samples were measured by high performance liquid chromatography

following a modified version of a previously published method (Pedruzzi et al., 2007). The liquid chromatography system (Agilent 1200, Agilent Technologies Inc., California, USA) used for analysis was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., California, USA) coupled to a refractive index detector. Sulfuric acid (0.450 mM, pH 3.1) was employed as the mobile phase at a flow rate of 0.3 mL/min with the column temperature set at 75 °C. Data acquisition and analysis were performed with ChemStation software (Agilent).

3. Results and discussion

3.1. Influence of oxygen deprivation in a two-stage cultivation process

As can be seen in Fig. 1, a lactobionic acid titer of 15.9 g/L with a volumetric productivity of 0.26 g/L h was obtained by applying an oxygen deprivation following the growth phase (at 10 h). It can be seen from the lactobionic acid synthesis profile that its titer remained practically unchanged from 24 h right up to the end of the process, coinciding with the total absence of dissolved oxygen in the fermentation broth. In addition, a poor bioconversion efficiency of 37% was obtained, leaving 26.5 g/L of residual lactose after 60 h. The pH profile became un-asymmetric after switching directly from aerobic to an imposed oxygen-depleted production phase in which a rapid drop in the concentration of dissolved oxygen from 80% to 30% was observed within less than 40 min, remaining at <1% from 20 h onwards. Accordingly, pH peaked at 10 h after completing the growth phase and its profile showed an unusual gradual drop associated with a delayed production rate of lactobionic acid under oxygen deprivation conditions. These results show that the oxidative bioconversion of lactose was inhibited by restricting the supply of oxygen, despite having attained a biomass concentration of 1.24 g/L after the aerobic phase.

3.2. Effect of the aeration rate on cell growth and lactobionic acid production from whey

Fig. 2 shows the impact of different oxygen supply levels on bioprocess parameters and lactobionic acid production from whey by *P. taetrolens*. Lower aeration rates contributed to enhanced cell growth of *P. taetrolens*, the highest maximum biomass concentration (1.36 g/L) being obtained under the influence of 0.5 Lpm (Fig. 2A). Similar maximum biomass values of 1.25 and 1.22 g/L were obtained under aeration rates of 1 and 1.5 Lpm, respectively. Conversely, the use of the highest oxygen supply scheme (2 Lpm) only supported a cell density of 1.16 g/L after the growth phase. The oxygen-limited conditions encountered by cells under aeration supply deprivation (0 Lpm) clearly constrained cell growth, yielding a maximum biomass of only 0.57 g/L.

Dissolved oxygen profiles were strongly related to the growth phase, a sharp depletion in dissolved oxygen concentration being found at the initial stage of the cell growth phase followed by a sudden rapid increase to saturated values indicating the onset of the production phase (Fig. 2B). In fact, rapid exhaustion of the dissolved oxygen was found under the absence of oxygen supply (0 Lpm), DOT remaining at <1% throughout the batch cultivation process. Furthermore, there was not a substantial difference in the minimum DOT levels obtained in the fermentation broth although the dissolved oxygen availability during growth phase was slightly increased with the aeration rate. Thus, a minimum DOT level of 5% was reached during growth phase at an aeration rate of 0.5 Lpm in comparison to 33% attained at 2 Lpm. In many bioprocesses, oxygen supply plays a key role in cellular growth and metabolite production due to the fact that poor volumetric mass transfer (K_La) becomes the rate-limiting factor as fermentation progresses (Wang et al., 2010; Lozano et al., 2011). However, as Fig. 2B shows, cultivations with aeration rates higher than 0.5 Lpm were not limited by dissolved oxygen availability. In the system under study, the highest aeration rate (2 Lpm) provided a K_La (49.98 ± 2.12 h^{-1}) 2-fold higher than the lowest aeration rate (0.5 Lpm), whereas medium aeration schemes such as 1 and 1.5 Lpm resulted in K_La values of 36.88 ± 1.31 and 44.05 ± 2.37 h^{-1}, respectively (Fig. 3). Obviously, K_La was affected by the different aeration rates employed, indicating the system capacity of oxygen supply.

As regards pH profiles, fermentation broths suffered the usual sharp increase in pH values during the exponential growth phase (Alonso et al., 2011), as shown in Fig. 2C. However, as a result of ineffective growth, cultivation under aeration supply deprivation (0 Lpm) showed an absence of pH-shift at the same time as reduced lactose bioconversion, remaining the pH profile unchanged and stable at 6.5 throughout the batch culture process (Fig. 2C). pH-shift values were correlated with aeration rates (Fig. 3), an increase in aeration rates leading to a progressive rise in pH-shift values (7.76, 8.00, 8.20 and 8.32 for 0.5, 1, 1.5 and 2 Lpm, respectively). Batch cultivation with an aeration rate of 2 Lpm showed the highest culture broth basicity, with a substantial increase in its duration (pH of 8.32 at 16.4 h), whereas the maximum pH value obtained when employing an aeration rate of 0.5 Lpm was only 7.76 at 9 h (Fig. 2C). Moreover, oxygen demand by *P. taetrolens* cells

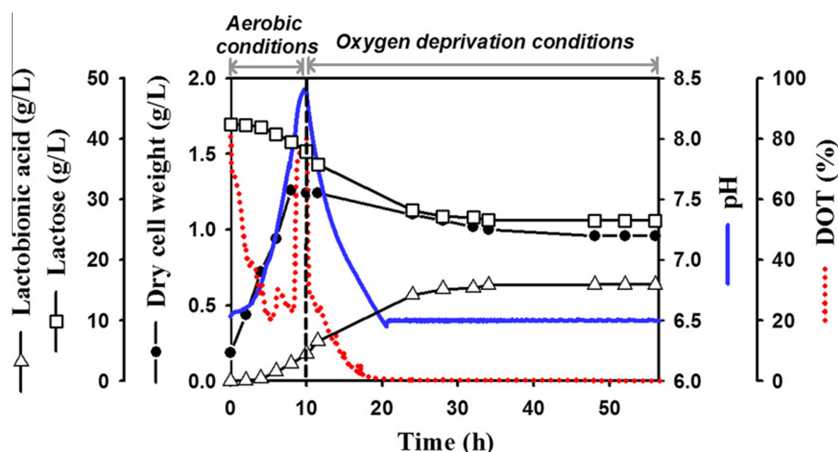

Fig. 1. Time-course profile of bioprocess parameters during pH-shift bioconversion under dissolved oxygen deprivation upon cellular growth phase. The short dashed vertical line marks the shut down of aeration at 10 h.

Fig. 2. Effect of aeration supply levels on *P. taetrolens* batch cultivations. Time-course profiles of cell growth (A), DOT (B), pH (C), OUR (D), lactose (E) and lactobionic acid (F) are shown.

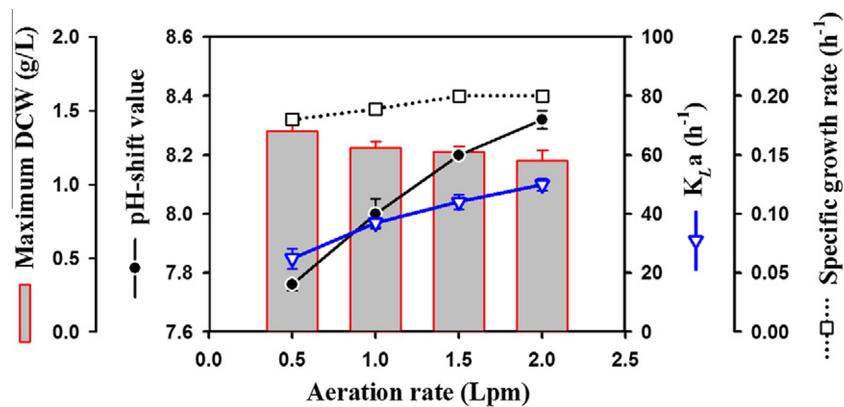

Fig. 3. Relationship between aeration rates and specific growth rate, maximum biomass, K_La and pH-shift values obtained during batch cultivations.

Table 1
Summary of values obtained in batch cultures under the influence of the aeration rate after cultivation.

Aeration rate (Lpm)	Maximum dry cell weight (g/L)	Duration of growth phase (h)	pH-shift value	μ (h^{-1})	Volumetric productivity (g/L h)	Culture time (h)	Yield (%)[a]
0	0.57 ± 0.06	12	–	0.08	0.04 ± 0.02	60	6 ± 2.5
0.5	1.36 ± 0.05	8	7.76 ± 0.02	0.18	0.80 ± 0.00	52	100 ± 0
1	1.25 ± 0.04	8	8.00 ± 0.05	0.19	0.70 ± 0.00	60	100 ± 0
1.5	1.22 ± 0.04	8	8.20 ± 0.00	0.20	0.70 ± 0.00	60	100 ± 0
2	1.16 ± 0.07	10	8.32 ± 0.03	0.20	0.54 ± 0.03	60	78 ± 3

–: absence of pH-shift value; μ = specific growth rate.
[a] Yield was defined as the percentage of lactose converted into lactobionic acid after culture time.

coincided with this increase in pH, higher maximum OUR values being obtained under increased oxygen supply conditions (Fig. 2D). Cultivation employing the highest aeration rate (2 Lpm) presented a maximum OUR value of 115 mg/L h at 8 h, compared to the 59.4 mg/L h obtained with 0.5 Lpm at 6 h. However, it was observed that the OUR decreased with the onset of the production phase due to nutrient limitation. Such diverse oxygen demands evidence that these cultivations were markedly influenced by the different volumetric oxygen transfer rates provided as a function of oxygen supply (Fig. 3). Changes in the OUR as well as K_La provided improved knowledge of this incipient biotechnological system, elucidating the role of dissolved oxygen within oxidative bioconversion.

The use of high aeration rates was detrimental to lactobionic acid production, as can be seen in Fig. 2F. Lactobionic acid production decreased markedly with increasing aeration rates. In fact, complete lactose bioconversion was achieved after 52 h at 0.5 Lpm compared to a 78% process yield obtained after 60 h under an aeration rate of 2 Lpm (Fig. 2F). Accordingly, optimal aeration rate for a complete lactose bioconversion ranged from 0.5 to 1.5 Lpm as Fig. 2E shows. Table 1 summarizes the overall cultivation parameters obtained with the five different aeration rates employed. A reduced specific growth rate (0.08 h^{-1}) as well as a low titer of lactobionic acid (2.5 g/L) were obtained under the absence of aeration (0 Lpm), suggesting that the lack of oxygen supply inhibited microbial cell proliferation and subsequent lactobionic acid synthesis (Fig. 2A and E). In fact, normal growth patterns as well as metabolite synthesis are negatively affected by the unbalanced redox state of cells generated under oxygen deprivation conditions in many bioprocesses involving aerobic microorganisms (Huang et al., 2006). Nevertheless, *P. taetrolens* displayed a broad tolerance to oxygen, as reflected by the fact that it grew under oxygen supply deprivation conditions, although only a maximum biomass of 0.57 g/L was obtained after 60 h. Conversely, these microaerobic conditions barely influenced lactose oxidative bioconversion (Fig. 2E), seeing as the yield was reduced up to 6% (Table 1).

3.3. Effect of the agitation rate on bioprocess parameters

The time courses of the bioprocess parameters under the influence of different agitation rates (at a constant aeration rate of 1 Lpm) are shown in Fig. 4. As can be seen, higher agitation rates stimulated growth of *P. taetrolens* cells, resulting in improved proliferation patterns, maximum biomass values of 1.06, 1.25, 1.29, 1.40 and 1.48 g/L being progressively obtained for 150, 350, 500, 700 and 1000 rpm, respectively (Fig. 4A). As can be seen in Fig. 4B, culture broths were supplied with different dissolved oxygen levels by changing the agitation speed in order to meet the oxygen demands of microbial cells. Thus, full aeration conditions were encountered by *P. taetrolens* cells under agitation rates higher than 700 rpm (Fig. 4B). At 150 rpm, the oxygen supplied was completely consumed by *P. taetrolens*, thus leading to a microaerobic environment (DOT < 1%) in the fermentation broth for 10 h. Results from specific growth rates also revealed that higher agitation rates stimulated cell growth performance. The specific growth rate thus increased with increasing agitation rate, although a similar rate of around 0.20 h^{-1} was found for medium (350, 500 rpm) and high (700, 1000 rpm) agitation regimes (Fig. 5). Regarding the onset of the production phase, the same duration of growth phase (8 h) was found for cultivations with agitation regimes higher than 350 rpm. The onset of the production phase at the lowest rate (150 rpm) was delayed, however, due to the oxygen-limited conditions (DOT < 1% for ~10 h) encountered by *P. taetrolens* cells, which clearly suffered a reduced proliferation (0.12 h^{-1}) during the growth phase compared to those obtained at higher rates (>0.20 h^{-1}).

Parallel to this decrease in dissolved oxygen, pH profiles showed an increase during the growth phase, as shown in Fig. 4C. The diverse dissolved oxygen availability explained the different pH-shift values observed during batch cultivations, being 7.88, 8.00, 8.10, 8.23 and 8.45 for 150, 350, 500, 700 and 1000 rpm, respectively. Temporal distribution of these pH-shifts, with the exception of that from cultivation at 150 rpm, took place at 8 h, coinciding with the end of the growth phase (Table 2). Microbial oxygen requirements and consumption also increased with the agitation rate, as Fig. 4D shows. The onset of the production phase featured a gradual decrease in the OUR, suggesting that the maximum metabolic activity of the cells was reached during the growth phase, subsequently remaining constant in the latter stage of fermentation. Moderate agitation regimes thus resulted in a lower dissolved oxygen content being available for the microbial metabolism of *P. taetrolens* cells, leading to either a reduction in pH-shift values or the OUR. Furthermore, vigorous agitation regimes (700 and 1000 rpm) provided batch cultivations with a significantly higher K_La, which resulted in 7- and 7.5-fold higher values compared to the lowest agitation rate (150 rpm, 10.63 ± 1.12 h^{-1}). However, K_La levels were fixed at 36.88 ± 1.31 and 56.80 ± 1.62 h^{-1} for medium agitation rates (350 and 500 rpm), as can be seen in Fig. 5. It is clear that increasing agitation facilitated cell proliferation by enhancing overall oxygen mass transfer. Thus, the detrimental effect of oxygen depletion at 150 rpm lay in oxygen mass transfer limitations due to the fact that poor mixing caused ineffective oxygen transfer and, as a result, the inefficient availability of dissolved oxygen for microbial cells.

Despite the fully aerobic conditions obtained at agitation rates above 500 rpm (Fig. 4B), lactobionic production was not related to oxygen availability, as Fig. 4F shows. In terms of lactobionic acid volumetric productivity, the best culture performance was obtained at 350 rpm, with a productivity of 0.70 g/L h, compared to the 0.57, 0.56 and 0.58 g/L h obtained at 150, 700 and 1000 rpm, respectively (Table 2). Similar process yields (of around 80%) were obtained for these three batch cultivations, although cultivation at 150 rpm exhibited retarded cell growth proliferation (Fig. 4A) and a lowered OUR (Fig. 4D) due to oxygen-limited conditions which clearly inhibited subsequent lactobionic acid production synthesis during the initial stage of the lactose oxidative process (Fig. 4E). Hence, medium agitation rates (350 and 500 rpm) were found to

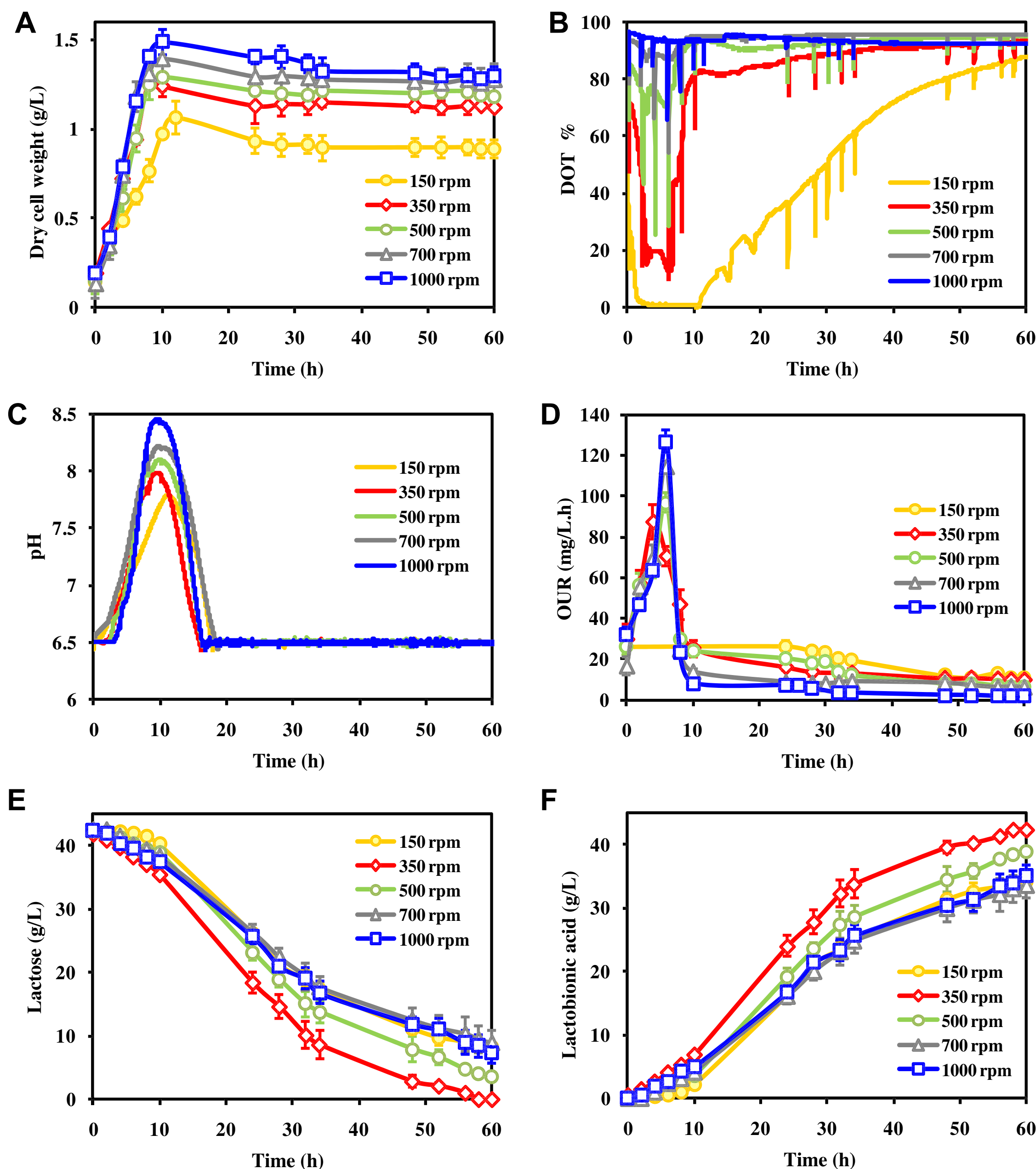

Fig. 4. Time-course profiles of biomass (A), DOT (B), pH (C), OUR (D), lactose (E) and lactobionic acid (F) at different agitation rates in pH-shift bioreactor cultivations with an aeration rate of 1 Lpm.

be the best oxygen supply conditions for lactobionic acid production despite obtaining lower cell densities than those at vigorous rates (700 and 1000 rpm) after the growth phase.

3.4. Influence of the dissolved oxygen level under high-cell density conditions

In order to further clarify the role of oxygen on lactobionic production from whey by *P. taetrolens* cells, cultivations were carried out under high-cell density conditions. The influence of different dissolved oxygen levels on the bioprocess parameters is shown in Fig. 6 and summarized in Table 3. In terms of cell growth, cultivations carried out under high oxygen supply conditions (40% and 60%) showed slightly enhanced maximum cell densities (1.45 versus 1.35 g/L). Likewise, a longer cell growth phase (6 h) was obtained at a level of 60% compared to 4 h obtained at lower levels. In response to increased oxygen availability, *P. taetrolens* cells displayed better specific growth rates and cultivations presented higher pH-shift values. However, as can be seen in Fig. 6, dissolved oxygen availability was detrimental for lactobionic acid production, as the lactobionic acid yield was progressively reduced under high supply conditions, yielding complete lactose bioconversion under non-controlled conditions (DOT < 5% during the growth phase) and at a level of 20%. The influence of dissolved oxygen availability shows that lactobionic acid synthesis by *P. taetrolens* cells increased markedly when cultivations were submitted to a short oxygen-limited period (DOT between 0–20%). Fermentation efficiencies in terms of volumetric productivity under oxygen-limited conditions were also enhanced, being 1.7- and 1.5-fold (1.27 and 1.12 g/L h for non-controlled and the 20% level, respectively) higher than under the highest oxygen supply scheme (0.75 g/L h for a level of 60%). In fact, oxygen limitation can induce

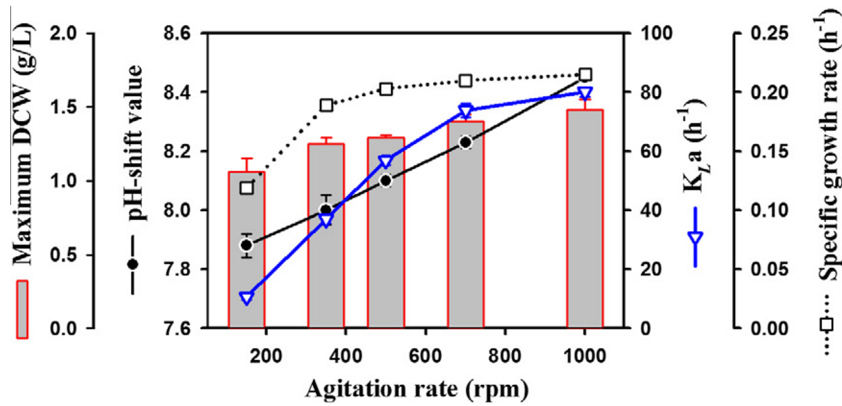

Fig. 5. Relationship between agitation rates and specific growth rate, maximum biomass (expressed as Dry Cell Weight, DCW), K_La and pH-shift values obtained during batch cultivations.

Table 2
Summary of values obtained under the influence of the agitation rate on batch production of lactobionic acid from whey after 60 h.

Agitation rate (rpm)	Maximum dry cell weight (g/L)	Duration of growth phase (h)	pH-shift value	μ (h^{-1})	Volumetric productivity (g/L h)	Yield (%)[a]
150	1.06 ± 0.09	10	7.88 ± 0.04	0.12	0.57 ± 0.02	82 ± 3
350	1.25 ± 0.04	8	8.00 ± 0.05	0.19	0.70 ± 0.00	100 ± 0
500	1.29 ± 0.02	8	8.10 ± 0.02	0.20	0.65 ± 0.00	93 ± 0
700	1.40 ± 0.05	8	8.23 ± 0.02	0.21	0.56 ± 0.03	80 ± 4
1000	1.48 ± 0.07	8	8.45 ± 0.00	0.21	0.58 ± 0.03	82 ± 4

μ = specific growth rate.
[a] Yield was defined as the percentage of lactose converted into lactobionic acid after 60 h.

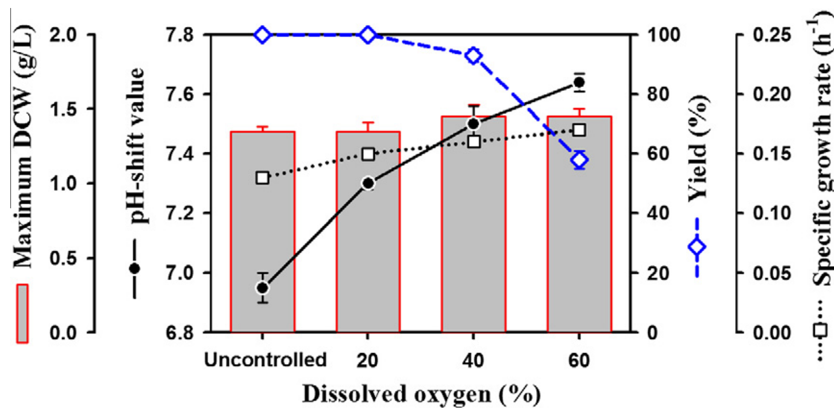

Fig. 6. Effects of different dissolved oxygen levels on bioprocess parameters under high-cell density conditions.

Table 3
Summary of values obtained in high-cell density batch cultivations in which DOT was controlled at different levels via an agitation cascade (from 350 to 500 rpm).

DOT level (%)	Maximum dry cell weight (g/L)	Duration of growth phase (h)	pH-shift value	μ (h^{-1})	Volumetric productivity (g/L h)	Culture time (h)	Yield (%)[a]
Not controlled	1.35 ± 0.03	4	6.95 ± 0.05	0.13	1.27 ± 0.00	30	100 ± 0
20	1.35 ± 0.06	4	7.30 ± 0.02	0.15	1.12 ± 0.00	32	100 ± 0
40	1.45 ± 0.08	4	7.50 ± 0.06	0.16	1.10 ± 0.02	32	93 ± 2
60	1.45 ± 0.05	6	7.64 ± 0.03	0.17	0.75 ± 0.04	32	58 ± 3

μ = specific growth rate.
[a] Yield was defined as the percentage of lactose converted into lactobionic acid after culture time.

secondary metabolite production (Clark et al., 1995), although, as previously shown, prolonged oxygen depletion in this fermentation system can constitute a limiting factor for cell growth and subsequent lactobionic acid production from whey by *P. taetrolens* due to the delayed onset of the production phase.

Interestingly, lactobionic acid titers obtained were 2.9-fold lower than those attained by Pedruzzi et al. (2011) who reported around 125.4 g/L of lactobionic acid from 252 g/L of chemically pure lactose solution employing permeabilized resting cells of *Zymomonas mobilis*. Conversely, this later biotransformation sys-

tem was featured by a specific productivity of around 0.8 g/g DCW h which was substantially lower than that obtained in the present work under high-cell density conditions (0.94 g/g DCW h), representing thus an efficient rate of bioconversion ability by *P. taetrolens* cells.

4. Conclusions

Results have revealed how dissolved oxygen availability plays a key role in the overall efficiency of the system under study. Poor bioconversion performances were obtained under vigorous agitation rates, which did however trigger cell proliferation of *P. taetrolens*. Conversely, the use of high aeration rates neither stimulated cell growth nor lactobionic acid production due to a prolonged proliferation phase that delayed the onset of the production phase. Therefore, addressing the deleterious influence of high oxygen supply schemes constitutes valuable information for industrial-scale bioproduction of lactobionic acid from whey.

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4.5. *Influencia de la heterogeneidad fisiológica sobre la producción de ácido lactobiónico*

No sólo los gradientes de concentración (hidrodinámicos, de sustrato, etc…) influyen decisivamente sobre el correcto devenir de un bioproceso en términos productivos (como han recogido de forma excelente Lara et al. (2006)) sino como Enfors et al. (2001) han señalado magnificamente, las respuestas fisiológicas de los microorganismos (como consecuencia de la falta de homogeneidad) pueden jugar un papel clave en el desarrollo de todo sistema fermentativo a escala industrial. Es indudable que la heterogeneidad fisiológica juega un papel clave en todo bioproceso, incluso dicho concepto está en la génesis de la falta de adaptación microbiana o en la propia aparición de fases lag. En el presente trabajo, se ha estudiado por primera vez la heterogeneidad fisiológica subyacente como factor inherente o implícito al desarrollo del proceso de producción de ácido lactobiónico por *P. taetrolens* a partir de suero lácteo en condiciones naturales (sin control de pH y sin aporte de oxígeno, existiendo por lo tanto una limitación en el nivel de oxígeno disuelto) en contraposición con las condiciones generadas en un sistema bajo control de pH (*pH-shift strategy*). Para llevar a cabo tales conclusiones se han desarrollado y optimizado dos protocolos dual-paramétricos de citometría de flujo basados en la actividad metabólica, la integridad de la membrana y en la polarización de la misma. Además, se ha desarrollado una estrategia de separación celular (*fluorescence-activated cell sorting*, FACS) en placa de agar (con un *screening* oxidativo simultáneo) llegando a poder discriminar y concluir que la gran mayoría de las células dañadas (el 75%) se corresponde con células viables pero no cultivables (células *viable but not culturable*, VBNC). Asimismo, se ha descrito por primera vez (hasta ahora tal enfoque no había sido desarrollado en ningún sistema fermentativo o cultivo) cómo la edad del inóculo (asociada a una heterogenidad fisiológica subyacente) conlleva la aparición de fases de latencia, la falta de adaptación o de proliferación celular de un sistema fermentativo. Así, la presencia de células muertas o dañadas como subpoblaciones dominantes ha conllevado cambios más que significativos en los valores asociados al rendimiento y productividad. De este trabajo se puede concluir que el enfoque clásico de optimización de todo proceso fermentativo, basado en el seguimiento de determinadas variables macroscópicas, debe considerar la información aportada por determinados parámetros fisiológicos con el fin de concocer el estado de salud real (*health status*).

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Physiological heterogeneity of *Pseudomonas taetrolens* during lactobionic acid production

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Abstract Physiological heterogeneity constitutes a critical parameter in biotechnological systems since both metabolite yield and productivity are often hampered by the presence of undesired physiological cell subpopulations. In the present study, the physiological status and functionality of *Pseudomonas taetrolens* cells were monitored by multiparameter flow cytometry during fermentative lactobionic acid production at the shake-flask and bioreactor scale. In shake-flask fermentation, the onset of the lactobionic acid production phase was accompanied by a progressive loss of cellular metabolic activity, membrane polarization, and membrane integrity concomitantly to acidification. In fact, population dynamics has shown the prevalence of damaged and dead subpopulations when submitted to a pH<4 from 16 h onwards. Furthermore, fluorescence-activated cell sorting revealed that these sublethally injured cells were nonculturable. In contrast, *P. taetrolens* cells exhibited a robust physiological status during bioreactor cultivations performed with a pH-shifted strategy at 6.5, remaining predominantly healthy and metabolically active (>96 %) as well as maintaining bioconversion efficiency throughout the course of the fermentation. Additionally, an assessment of the seed culture's physiological robustness was carried out in order to determine the best seed culture age. Results showed that bioreactor culture performance, growth, and lactobionic acid production efficiency were strongly dependent on the physiological heterogeneity displayed by the seed culture. This study provides the most suitable criteria for optimizing lactobionic acid production efficiency through a novel flow cytometric-based approach based on the physiological status of *P. taetrolens*. It also constitutes a valuable, broad-ranging methodology for the enhancement of microbial bioprocesses involved in the production of secondary metabolites.

Keywords Flow cytometry · Physiological status · Lactobionic acid · Whey · *Pseudomonas taetrolens* · Secondary metabolite

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Introduction

Both overall efficiency and metabolite productivity within a bioprocess are strongly reliant on the physiological status and functionality displayed by microbial cells. In fact, physiological heterogeneity constitutes a critical parameter in biotechnological systems since variations in cell capacities may lead to undesired cell subpopulations of reduced efficiency (Avery 2006; Müller et al. 2010). The segregation in the phenotype of an isogenic population due to unbalanced environmental or bioprocessing conditions (Amanullah et al. 2003; Nicolau et al. 2010) can undoubtedly result in poor culture fitness as well as reduced microbial performance (Zhang et al. 2009).

In many industrially relevant biotechnological processes, bioprocessing conditions often involve cellular stress responses with substantially deleterious effects on physiological status (Enfors et al. 2001; Zhu et al. 2011). Major cell responses have been found to be induced as a result of environmental perturbations such as lack of nutrient availability (Hewitt et al. 1999; Lopes da Silva et al. 2005), stressing (Amanullah et al. 2003; Amor et al. 2002; Papadimitriou et al. 2007), or hydromechanical conditions (Lüders et al. 2011). Depending on the nature or degree of these perturbations, loss of cellular functionalities could consequently result in irreversible cellular senescence (Bogosian and Bourneuf 2001; Nyström 2005). The

complex responses and performances of bacteria in biotechnological systems have therefore led to the development of advanced monitoring tools to achieve a better understanding of microbial physiology at the population level (Díaz et al. 2010; Hammes et al. 2011). While conventional bulk approaches for microbial biomass estimation reflect neither the functional information nor the physiological robustness of cells (Hewitt and Nebe-von-Caron 2001), multiparametric flow cytometry has emerged as a valuable technique to detect and monitor changes in these parameters. Such information on the *health status* of cells may explain cell-to-cell variability in behavior, robustness, shape, or size, since changes in these microbial parameters are intimately linked to the presence or absence of an intact fully polarized cytoplasmic membrane and the transport systems across it (Hewitt and Nebe-von-Caron 2004). Furthermore, assessment of the physiological status of cells using flow cytometry can reveal asynchronous phenotypic subpopulations comprising non- and metabolite-producing cells (David et al. 2011; Herrero et al. 2006) or even community dynamics within a bacterial consortium (Müller et al. 2009). Undoubtedly, a concise understanding of the underlying microbial physiology provides insights into the most suitable cultivation strategy for enhanced metabolite production, as well as unraveling the deleterious effects on cellular fitness (Lopes da Silva et al. 2009).

Although numerous studies have focused on flow cytometric assessment of bacteria (Díaz et al. 2010; Hammes et al. 2011), only a few approaches to bioprocess monitoring involving *Pseudomonas* species have been developed to date (Amanullah et al. 2003; Nielsen et al. 2009). Considering the relevance of the genus *Pseudomonas* as a cell factory, the development and application of flow cytometry-derived information could be a valuable tool for enhanced biological performance of incipient bioprocesses. Bioproduction of lactobionic acid from an inexpensive source such as cheese whey through a fermentation-based process carried out by *Pseudomonas taetrolens* has thus become both a promising and attractive alternative to obtain this value-added substance (Alonso et al. 2011, 2012). Lactobionic acid has recently emerged as a relevant commercial polyhydroxy acid which displays interesting pharmaceutical properties with promising biomedical applications (Lin et al. 2011; Zhang et al. 2011). This versatile bioproduct also finds a broad range of applications in the cosmetics industry, in which it is being currently employed as the key active component in novel skin care products (Green et al. 2009). In view of the relevance of lactobionic acid bioproduction, significant improvements in microbial performance could be achieved after revealing the underlying microbial physiology and understanding the complex interaction between *P. taetrolens* cells and their surrounding microenvironment.

In this context, the aim of this study was to gain a deeper process understanding of the physiological status of *P. taetrolens* during fermentative lactobionic acid production at the shake-flask and bioreactor scale, highlighting the importance of the uncontrolled pH versus pH shift conditions encountered by the cells in each system. Cellular parameters such as membrane polarization, membrane integrity, and metabolic activity of *P. taetrolens* cells were evaluated and monitored through a multiparameter flow cytometry assessment in order to determine whether the physiological age of cells could impair lactobionic acid production from whey. The underlying physiology and population behavior of *P. taetrolens* were thus deciphered for the first time, thereby providing additional key information for the implementation of strategies to prevent potential deleterious bioprocessing effects on fermentation performance. From a practical point of view, information obtained from this study could also be of substantial interest for industrially relevant microbial systems submitted to stressing conditions involved in the production of secondary metabolites.

Materials and methods

Microorganism

P. taetrolens LMG 2336, obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium), was maintained frozen (in 40 % [*v*/*v*] glycerol at −20 °C). This strain was subsequently subcultured on nutrient broth (NB, containing 1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone, and 5 g/L NaCl) agar plates, incubated for 48 h at 30 °C, and then preserved at 4 °C.

Inoculum preparation

A loopful of *P. taetrolens* from a fresh nutrient broth agar plate was used to inoculate a 500-mL Erlenmeyer flask containing 100-mL of nutrient broth medium. This flask was incubated on an orbital shaker (Infors HT, model Flyer Aerotron, Switzerland) at 250 rpm and 30 °C for 10 h. Actively growing cells from this culture were then employed as inoculum for the production of lactobionic acid in shake-flask and bioreactor seed cultures containing sweet whey.

Sweet whey preparation

Sweet cheese whey (provided by ILAS S.A., Asturias, Spain) was 1-fold diluted with distilled water (1:1) and adjusted to pH 6.5 (by adding NaOH 6 N) prior to sterilization using a tangential microfiltration device equipped with a 0.22-μm pore size PVDF membrane cassette (Millipore, MA, USA).

Shake-flask fermentations

Shake-flask fermentation experiments were conducted in 500 mL Erlenmeyer flasks containing 100 mL of sweet whey inoculated with 0.64 g/L of wet biomass from NB inoculum cultures harvested by centrifugation at 11,000×*g* for 10 min. These cultures were subsequently incubated on an orbital shaker (New Brunswick Scientific, NJ, USA) at 250 rpm and 30 °C. Samples were aseptically withdrawn periodically to determine bacterial growth and pH. Biomass was removed by centrifugation at 16,000×*g* for 5 min, and the cell-free supernatants being stored frozen (−20 °C) until further analysis. Bioconversion experiments were carried out in triplicate as independent trials.

Bioreactor cultivations

Batch cultivations for lactobionic production from sweet whey were also performed in a 2-L bioreactor (BioFlo 110, New Brunswick Scientific, NJ, USA) with a working volume of 1 L. Bioreactor experiments were conducted with an inoculation level of 10 % (*v*/*v*), an agitation rate of 350 rpm, and at 30 °C. Moreover, the bioreactor was aerated (1.0 vvm) through a ring sparger located at the bottom of the vessel. Excessive foam formation was prevented by automatic addition of diluted (1:10) Y-30 emulsion (Sigma-Aldrich, Steinheim, Germany). The bioreactor was equipped with a pH meter (Mettler Toledo, Switzerland) and a polarographic dissolved oxygen electrode (InPro 6830, Mettler Toledo, Switzerland) for continuous on-line monitoring of pH and dissolved oxygen tension (DOT) values, respectively. A two-stage pH-shifted bioconversion strategy was adopted in the bioreactor experiments as previously reported (Alonso et al. 2011). The strategy consisted in controlling pH above 6.5 (pH was left uncontrolled above this value during the growth phase and then maintained at 6.5) by means of computer-controlled peristaltic pumps via automatic addition of 2 M NaOH. Cultivations were carried out in duplicate as independent experiments.

The seed culture was obtained by harvesting biomass (after centrifugation at 11,000×*g* for 10 min) from a NB inoculum culture and re-suspending cells in a 500-mL Erlenmeyer flask containing 100 mL of sweet whey. Subsequently, these cultures were grown at 250 rpm in an orbital shaker at 30 °C for 12 (control experiment, also used for the sake of comparison with shake-flask cultivation), 24, 36, or 48 h. Each culture was then employed as seed culture at 10 % (*v*/*v*) in the bioreactor containing sweet whey in order to study the influence of cellular physiological age on bioprocess performance.

Staining procedures

Samples from cultures were harvested by centrifugation at 16,000×*g* for 5 min. Before staining, cells were washed twice in phosphate-buffered saline (PBS, pH 7.4, sterile and filtered at 0.22 μm), and then cells were held in the "hot spot" of a sonication bath for 2 s in order to prevent bacterial aggregation before flow cytometric analysis (Hewitt and Nebe-von-Caron 2004). Propidum iodide (PI, Invitrogen), bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol, $DiBAC_4(3)$, Invitrogen), and carboxyfluorescein diacetate (cFDA, Invitrogen) were used as fluorescent dyes in a double dual-staining procedure ($DiBAC_4(3)$/PI and cFDA/PI) in order to evaluate cell physiological status (metabolic activity, membrane integrity, and membrane polarization were evaluated through cFDA, PI, and $DiBAC_4(3)$ staining, respectively). Stock solutions were prepared as follows: PI was made up to 1 mg/mL in distilled water (0.22 μm filtered) and maintained at 4 °C, whereas both $DiBAC_4(3)$ and cFDA were prepared in dimethyl sulfoxide (Sigma-Aldrich) at a concentration of 1 mM and stored at −20 °C. PI staining was prepared by diluting the stock solution in sterile distilled water and adding this work solution to the cell suspension at a final concentration of 5 μg/mL. This mixture was then incubated for 30 min in the dark at room temperature. Working solutions of $DiBAC_4(3)$ and cFDA were made up to 25 and 10 μM in PBS containing 1 mM EDTA, respectively. PI-stained samples were subsequently incubated with 1 μM $DiBAC_4(3)$ or 0.1 μM cFDA for 15 min in the dark at room temperature.

Multiparameter flow cytometry

Flow cytometry measurements were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with a 488- and 633-nm excitation light source from an argon ion laser. Green fluorescence from samples (corresponding to $DiBAC_4(3)$ and cFDA-stained cells) was collected on the FL1 channel (530 nm), whereas PI fluorescence was registered on the FL3 channel (610 nm). Each analysis was performed in duplicate at a low flow rate setting (4,000 events/s). Data acquisition was carried out using Cytomics RXP software (Beckman Coulter). Gates and quadrants were established according to staining controls. For $DiBAC_4(3)$/PI and cFDA/PI dual parameter flow cytometric analysis, data collected from 150,000 and 100,000 events, respectively, were analyzed using Summit v4.3 software (DakoCytomation, CO, USA).

Fluorescence-activated cell sorting

Cell sorting was performed using a MoFlo XDP cell sorter (DakoCytomation) equipped with an air-cooled argon ion

laser emitting blue light at 488 and 630 nm (200 mW). The green fluorescence from cFDA-stained cells was detected through a 530-nm filter (FL1 channel), whereas the red fluorescence from the PI signal was collected on the FL3 channel (610 nm). Fluorescent beads (Flow-check Pro Fluorospheres, Beckman Coulter) with sizes of 10, 6, and 3 μm were used to check the fluidics system and flow cytometer's optical alignment (coefficient of variation, CV value, around 2 %). Samples were sorted in *purify* mode at a rate of 200 cells/s using Isoton II (Beckman Coulter) as the sheath fluid. The instrument also operated at a system pressure of 54 psi and was equipped with a nozzle size of 70 μm. Sorting gates were established on the basis of FL1 (cFDA(+)/PI (−) cells) versus FL3 fluorescence (cFDA(−)/PI(+) cells). Data acquisition was performed using Summit v5.0 software (DakoCytomation). Bacterial cells from gates were sorted onto agar plates containing lactose (10 g/L), peptone (2 g/L), yeast extract (1 g/L), K_2HPO_4 (0.2 g/L), bromophenol blue (0.08 g/L), and agar (15 g/L) with the aim of screening culturability and lactose-oxidizing ability by *P. taetrolens* cells, as previously reported (Alonso et al. 2011). Subpopulations of interest were thus sorted onto 96 spots on agar plates, which were then incubated for 96 h at 30 °C.

Fluorescence and confocal microscopy

Stained samples were also examined under a Leica TCS-SP2-AOBS confocal laser scanning microscope (Leica Microsystems Inc., Heidelberg, Germany) at excitation wavelengths of 488 and 568 nm with an emission wavelength of 530 (green fluorescence) or 630 nm (red fluorescence).

Analytical methods

Bacterial growth was measured spectrophotometrically as optical density at 600 nm (Shimazdu, UV 1203 model) after centrifugation of culture samples at 16,000×*g* for 5 min. Optical density data were converted to cell dry weight (expressed in grams per liter) using the corresponding previously obtained calibration curve. The lactobionic acid and lactose content of cell-free culture samples were measured by high performance liquid chromatography as reported previously by Alonso et al. (2011).

Determination of the oxygen uptake rate

The oxygen uptake rate (OUR) from bioreactor cultivations was determined via the dynamic method (Bandyopadhyay et al. 2009). Consequently, the OUR was obtained from the slope of the plot of dissolved oxygen concentration versus time following a momentary interruption of air supply to the bioreactor (Alonso et al. 2012). The specific consumption rate of oxygen (qO_2) was calculated by dividing the OUR value by the biomass concentration at each sampling point.

Results

Monitoring of the physiological status of *P. taetrolens* during shake-flask fermentation

Figure 1 shows the changes in the physiological status of *P. taetrolens* in terms of metabolic activity, membrane integrity, and polarization during shake-flask fermentation. While panels a and b of Fig. 1 both illustrate the dual flow cytometric assessment of green fluorescence versus red fluorescence ($DiBAC_4(3)$/PI and cFDA/PI, respectively), Fig. 1c shows representative dot plot diagrams of the side scatter (SSC) versus forward side scatter (FSC) signal obtained during cultivation. The upper left quadrant of dot plots in Fig. 1a shows depolarized cells ($DiBAC_4(3)$-positive; $DiBAC_4(3)$ enters cells only when the membrane potential is lost), while cells with esterase activity (cFDA-positive cells; cFDA is cleaved by the esterase activity inside the living cells, thus releasing a polar fluorescent portion which is unable to pass through the intact membrane) are shown in the upper left quadrant of Fig. 1b. Damaged cells (corresponding to cFDA(+)/PI(+) cells or $DiBAC_4(3)$(+)/PI (−) cells) are shown in the upper left quadrant (Fig. 1a) and in the upper right quadrant (Fig. 1b) for the $DiBAC_4(3)$/PI and cFDA/PI staining, respectively. Likewise, dead cells (PI-positive cells; PI can only cross the plasmatic membrane if it is permeabilized corresponding to those cells whose membranes are compromised) are shown in the upper right quadrant and in the lower right quadrant for the $DiBAC_4(3)$/PI (Fig. 1a) and cFDA/PI staining (Fig. 1b), respectively. Examination of these cytograms reveals that shake-flask cultivation displayed marked physiological heterogeneity in terms of metabolic status, membrane integrity, and cellular size. Results based on esterase activity and membrane integrity thus suggest that loss of metabolic activity was progressive, leading first to an intermediate "damaged" cell state and then irreversibly to cell death (Fig. 1b). According to Fig. 1a, cells also showed a poor *health status* in terms of membrane polarization at later cultivation stages (36 and 48 h) given that the presence of an intact, fully polarized cytoplasmic membrane represents a signal of cellular robustness (Amor et al. 2002; Hewitt and Nebe-von-Caron 2004). As can be seen in Fig. 1c, an increase in the intensity of the FSC was evidenced in the flow cytometric dot plots. Cells of *P. taetrolens* exhibited both higher relative cell size (forward scatter, FSC) and cell complexity signals (side scatter, 90°-angle scatter of blue laser light, SSC) with age.

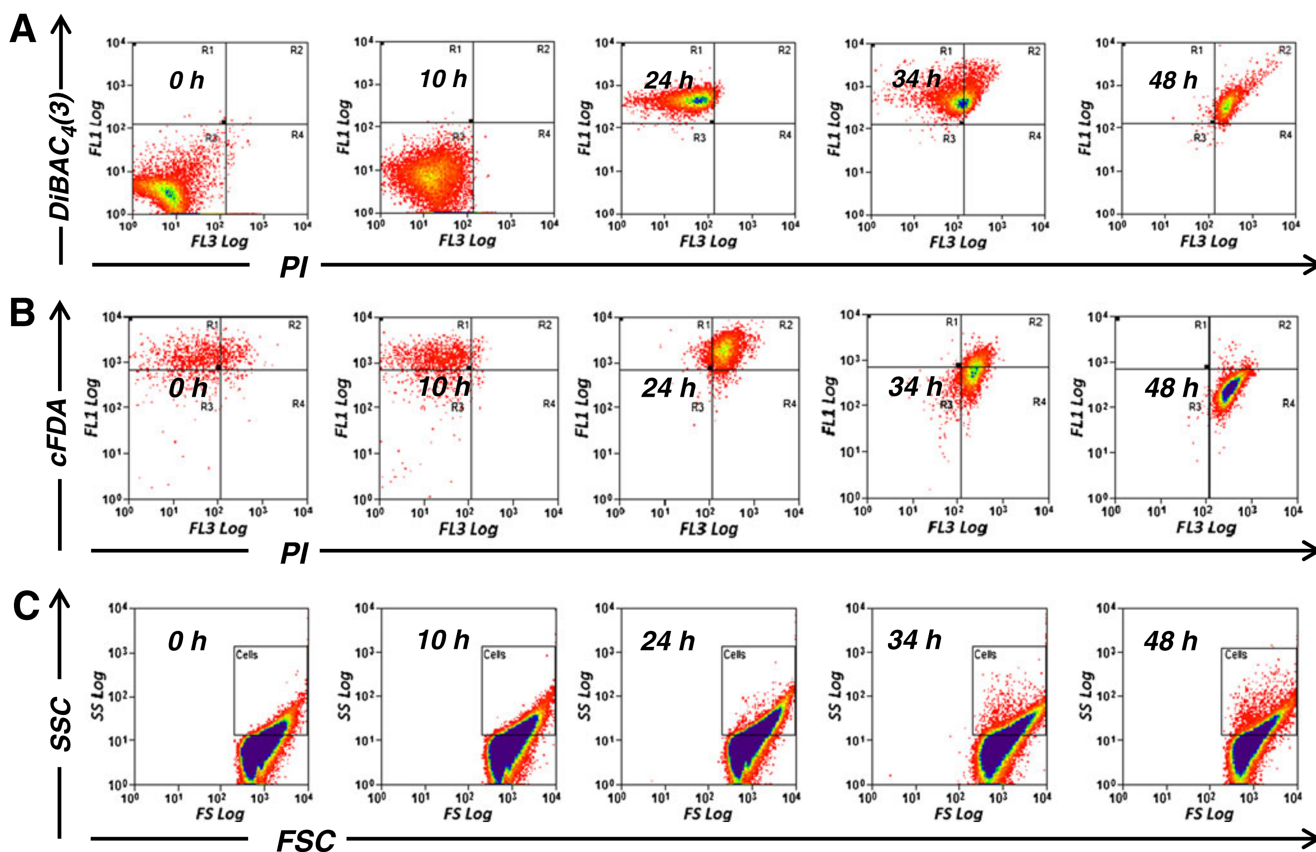

Fig. 1 Dot plots representing DiBAC$_4$(3) fluorescence versus PI fluorescence (**a**), cFDA fluorescence versus PI fluorescence (**b**), and side scatter light (SSC) versus forward scatter light (FSC) signals (**c**) of *P. taetrolens* cells during shake-flask fermentation

Figure 2a shows the bioprocess parameters obtained during shake-flask fermentation, which was featured by a specific lactobionic acid productivity of 0.38 g/g DCW h. These data provide evidence that lactobionic acid constitutes a secondary metabolite mostly produced by *P. taetrolens* during the stationary growth phase, as previously reported (Alonso et al. 2011). Cultivation reached the stationary growth phase after 10 h, coinciding with the onset of the production phase (Fig. 2a), reaching a maximum biomass of 1.84 g/L. During the growth phase, the pH first rose (with a pH shift value of 7.36 at 6 h) and then decreased abruptly, thereby signaling the onset of the production phase. A pH value of 3.42 was reached after 24 h, remaining unchanged at 3.35 from 48 h onwards, which coincided with the depletion of lactose content in the fermentation broth (Fig. 2a).

As can be seen in Fig. 2b, cells were predominantly healthy and metabolically active (>90 %) during the cell proliferation phase (0–10 h), simultaneously displaying an intact and polarized membrane. However, from 12 to 16 h, there was a sudden change in these parameters concomitant to the abrupt drop in pH from 6.2 to 4 and coinciding with the transition phase between growth and the lactobionic acid production phase (Fig. 2a, b). As a result, the population dynamics showed an increase in the percentage of positively DiBAC$_4$(3)-stained cells from 2 to 15 %. The onset of the lactobionic acid production phase was accompanied by the passing of healthy, metabolically active cells into a damaged state, which was accompanied by a progressive loss of membrane integrity and the predominance of cellular death from 36 h onwards (Fig. 2b), suggesting that the cells were highly affected by prolonged exposure to extreme acidic conditions (pH=3.35). Obviously, cells with a permeabilized cytoplasmic membrane cannot sustain the electrochemical gradient, leading irreversibly to cellular senescence (Díaz et al. 2010). Consequently, the lactobionic acid production phase in shake-flask cultivation was highlighted by a poor *health status* of *P. taetrolens* cells in terms of metabolic activity concomitant to cellular depolarization and an increase in the FSC signal.

Cell sorting at the single cell level

Cell sorting can reveal the relationship between the different subpopulations within a bacterial monoculture and their culturability (Papadimitriou et al. 2006; Want et al. 2011). Hence, with the aim of shedding more light

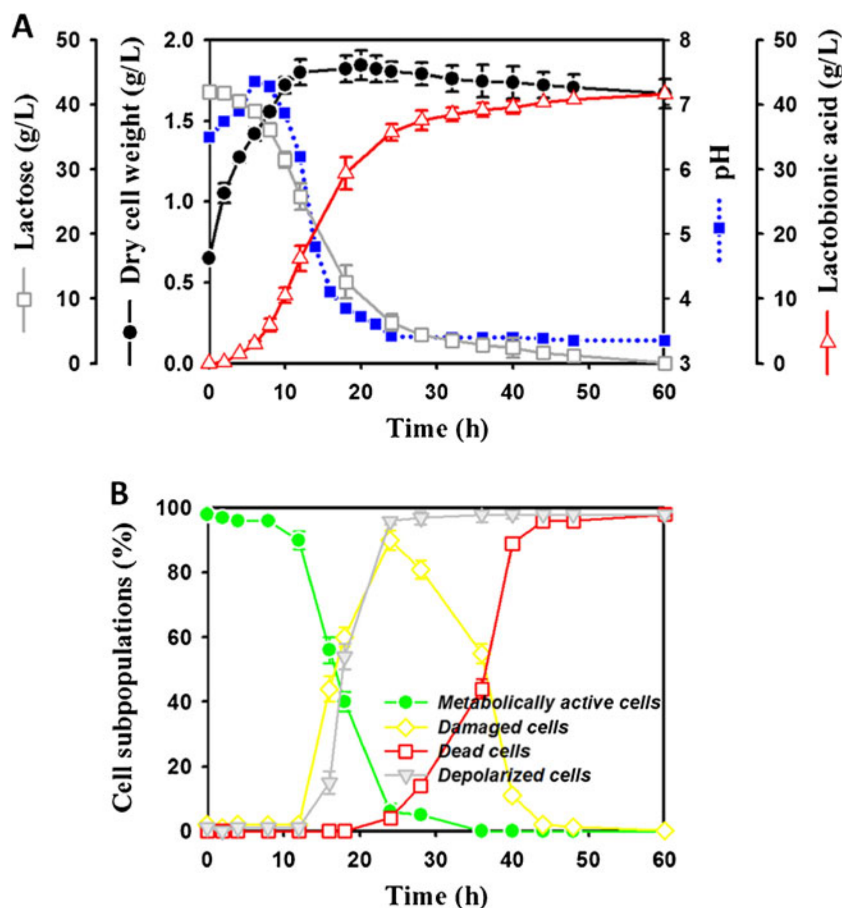

Fig. 2 Time-course profile of bioprocess parameters obtained during shake-flask cultivation of *P. taetrolens* (**a**). Percentages of *P. taetrolens* cell subpopulations throughout the shake-flask fermentation (**b**)

on the underlying physiology during shake-flask cultivation, *P. taetrolens* cells were sorted individually onto agar plates containing lactose. The observation of stained samples under fluorescence microscopy revealed that *P. taetrolens* cells were predominantly metabolically active at 12 h, whereas cells at the late stationary phase (36 and 48 h) were dead (Fig. 3a). As can be seen in Fig. 3a, aggregates of dead cells were detected and verified during the later stages of cultivation (36 and 48 h). Interestingly, the observed increase in light scattering properties during flow cytometric analysis (Fig. 1c) could be ascribed to this phenomenon, as previously reported by Kottmeier et al. (2009) for *Hansenula polymorpha* cells.

In accordance with the results obtained in Fig. 3b, cell sorting on agar plates demonstrated that the majority of cells at 12 h were culturable (97.9 %), while damaged cells (corresponding to the cFDA(+)/PI(+) cells found at 24 h) displayed a culturability of only 24.8 % (Fig. 3c). There was an abrupt decrease in the culturability of sorted cells (corresponding to cFDA(−)/PI(+) cells) at the late stationary growth phase (36 and 48 h), as no cell was capable of growing on agar plates (Fig. 3b, c), verifying that these cells were nonculturable and dead.

Monitoring of the physiological status of *P. taetrolens* during pH shift bioreactor cultivation

Flow cytometric dot plots obtained during the pH shift bioreactor cultivation are shown in Fig. 4. In accordance with the data, cells displayed a robust physiological status throughout the course of the fermentation (Fig. 4a, b). As Fig. 4b shows, cells exhibited high metabolic activity and retained their membrane integrity even at late stages of cultivation. The stationary phase was also accompanied by slight membrane depolarization, although the fluorescence intensity was significantly lower than that obtained in the shake-flask culture.

Figure 5a shows the bioprocess parameters obtained during pH shift bioreactor cultivation, which showed 1.5-fold higher specific lactobionic acid productivity (0.56 g/g DCW h) than that obtained in shake-flask cultivation. As can be seen, the growth phase was featured by an abrupt drop in the DOT concomitant to a sharp increase in pH values (pH shift of 8 at 8 h). The biomass reached a maximum value of 1.26 g/L at the end of the exponential growth phase, with a specific growth rate of 0.19 h^{-1}. The onset of the production phase took place with a concurrent return of DOT to values near to air saturation level (indicating the end of the

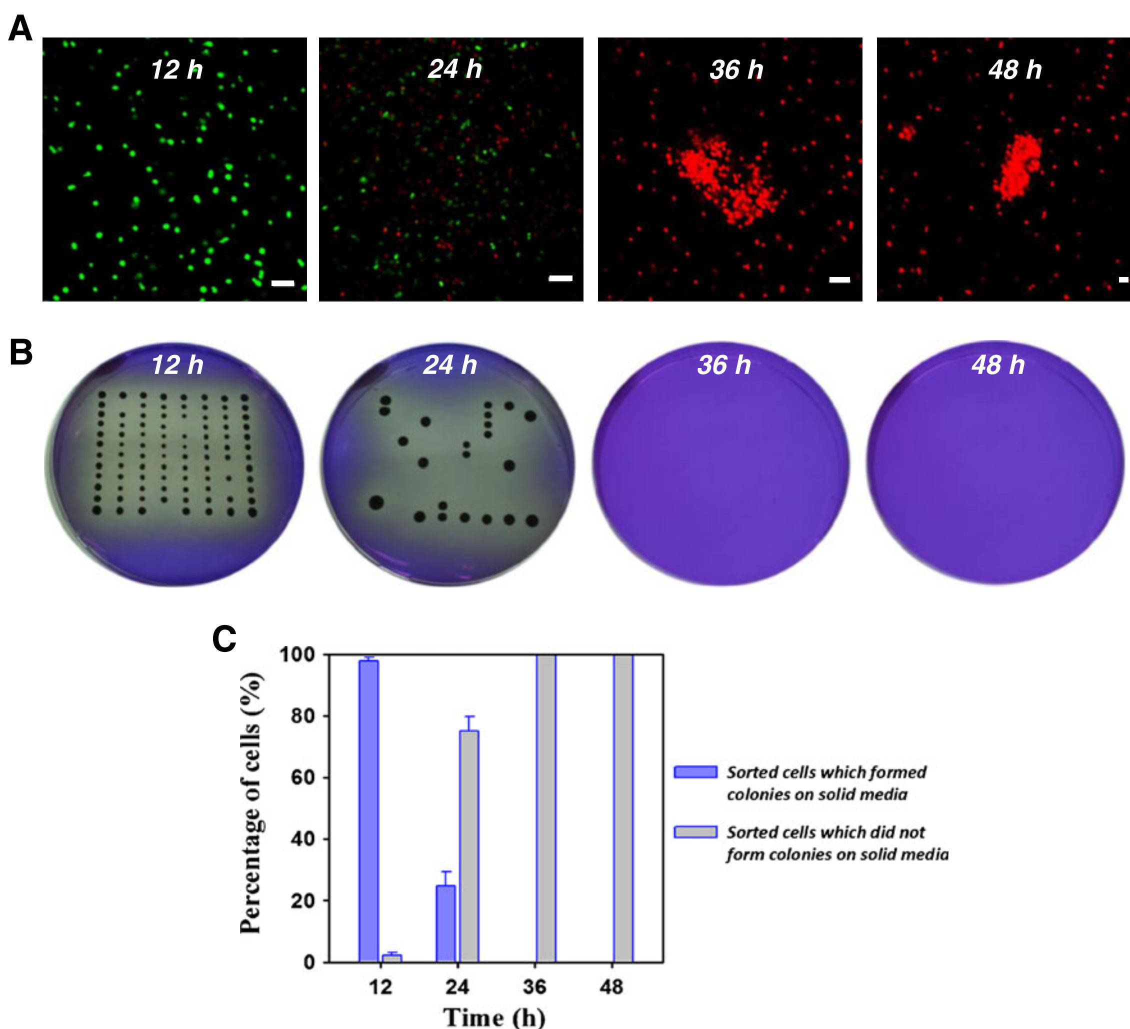

Fig. 3 Fluorescence micrographs of *P. taetrolens* cells from shake-flask fermentation stained with cFDA (green fluorescence) and PI (red fluorescence) (**a**). Aggregates of dead *P. taetrolens* cells were detected at later stages of cultivation (36 and 48 h). Culturability of stained cells from shake-flask cultivation at 12, 24, 36, and 48 h which were sorted directly on agar plates containing lactose (**b**). Percentages of cells that formed or did not form colonies on agar plates after fluorescence-activated cell sorting (**c**). *Scale bars*=5 μm

exponential cell growth phase). The profile of lactobionic acid production confirmed that lactobionic acid is a non-growth-related product, showing the typical production pattern from whey by *P. taetrolens* (Alonso et al. 2011, 2012). A maximum specific oxygen consumption rate (qO_2) of 156 mg/g h was obtained at the beginning of the fermentation process, subsequently remaining around 1.75 mg/g h during the stationary growth phase (Fig. 5a). Obviously, the sudden decrease in the DOT level during the first 8 h may be attributed to the increase in oxygen consumption resulting from the active growth proliferation of *P. taetrolens* cells.

Analysis of the physiological status of the cells revealed that 96 % of these remained healthy, metabolically active, and polarized during the cell proliferation phase (Fig. 5b). Upon shifting to the production phase, no significant changes were found in terms of metabolic activity (~96 % of the cells).

Influence of the physiological age of cells on fermentation performance at the bioreactor scale

Figure 6 shows the comparative profiles of bioprocess parameters obtained in pH shift bioreactor cultivations employing 12-, 24-, 36-, and 48-h seed cultures with the aim of determining the influence of seed culture age. As can be seen in Fig. 6a, younger seed cultures contributed to enhanced growth of *P. taetrolens*, obtaining a maximum biomass concentration of 1.25, 1.07, 0.98, and 0.85 g/L for 12-, 24-, 36-, and 48-h seed cultures, respectively. Both cell growth and the specific growth rate were clearly impaired by the increase in seed culture age, as can be seen in Table 1. Moreover, large lag phases preceded microbial growth in batch cultivations inoculated with seed cultures older than 12 h (Fig. 7). This lack of growth caused a prolonged delay in the onset of the production phase: the

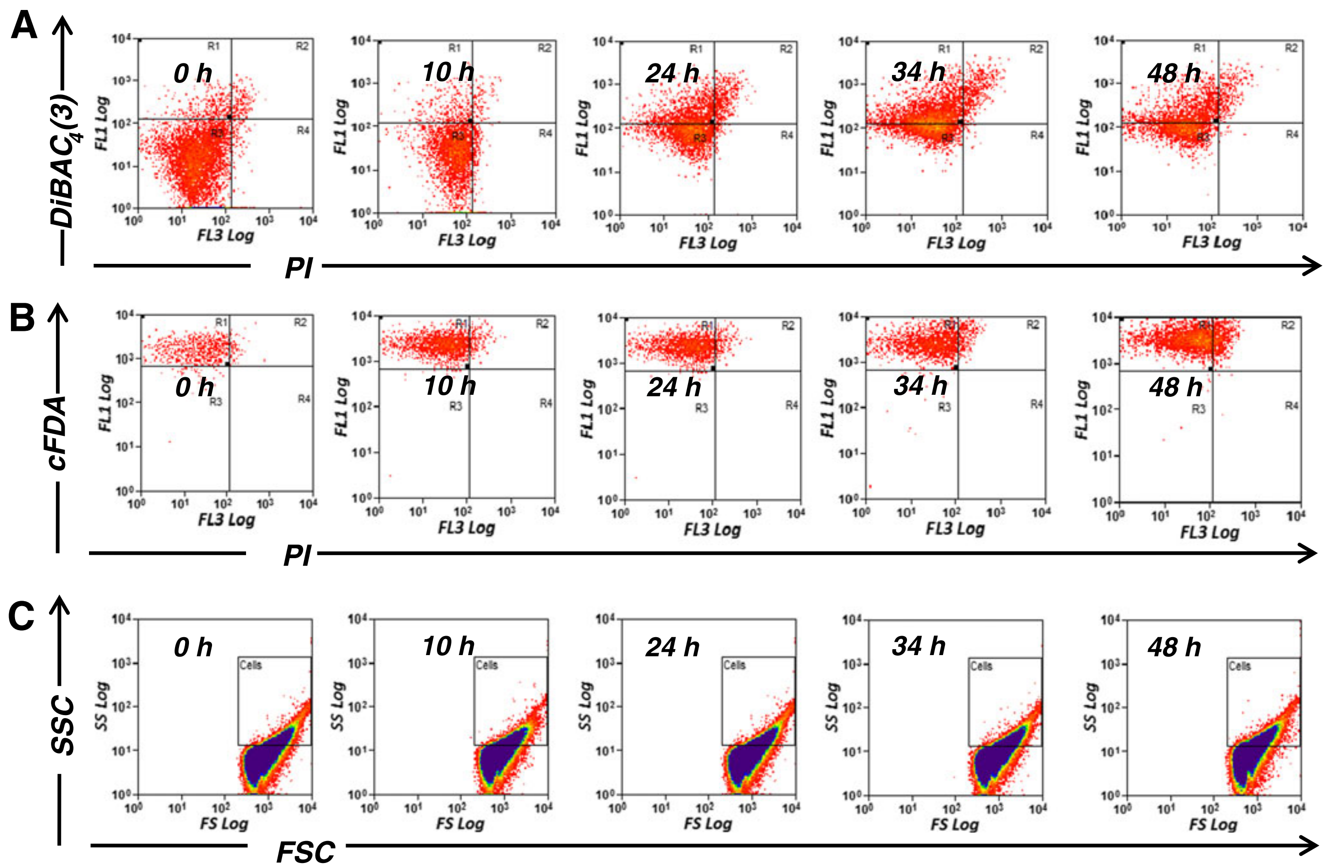

Fig. 4 Dot plots representing DiBAC$_4$(3) fluorescence versus PI fluorescence (**a**), cFDA fluorescence versus PI fluorescence (**b**), and light scattering properties (**c**) of *P. taetrolens* cells during pH shift bioreactor cultivation employing a seed culture age of 12 h

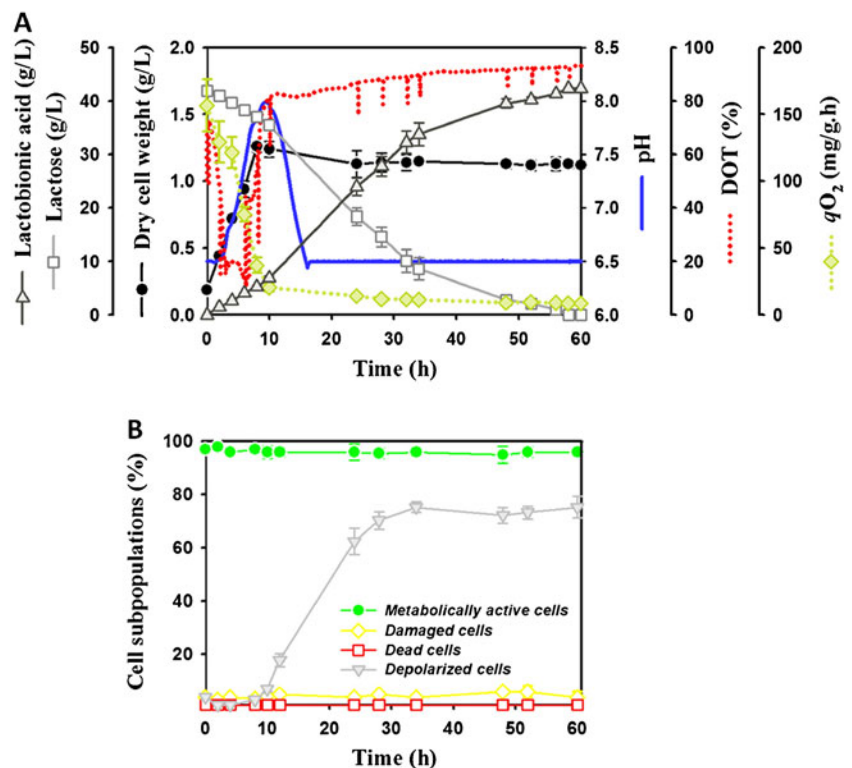

Fig. 5 Bioprocess parameters obtained during pH shift bioreactor cultivation of *P. taetrolens* (**a**). Percentages of *P. taetrolens* cell subpopulations throughout the bioreactor cultivation (**b**) employing a seed culture age of 12 h

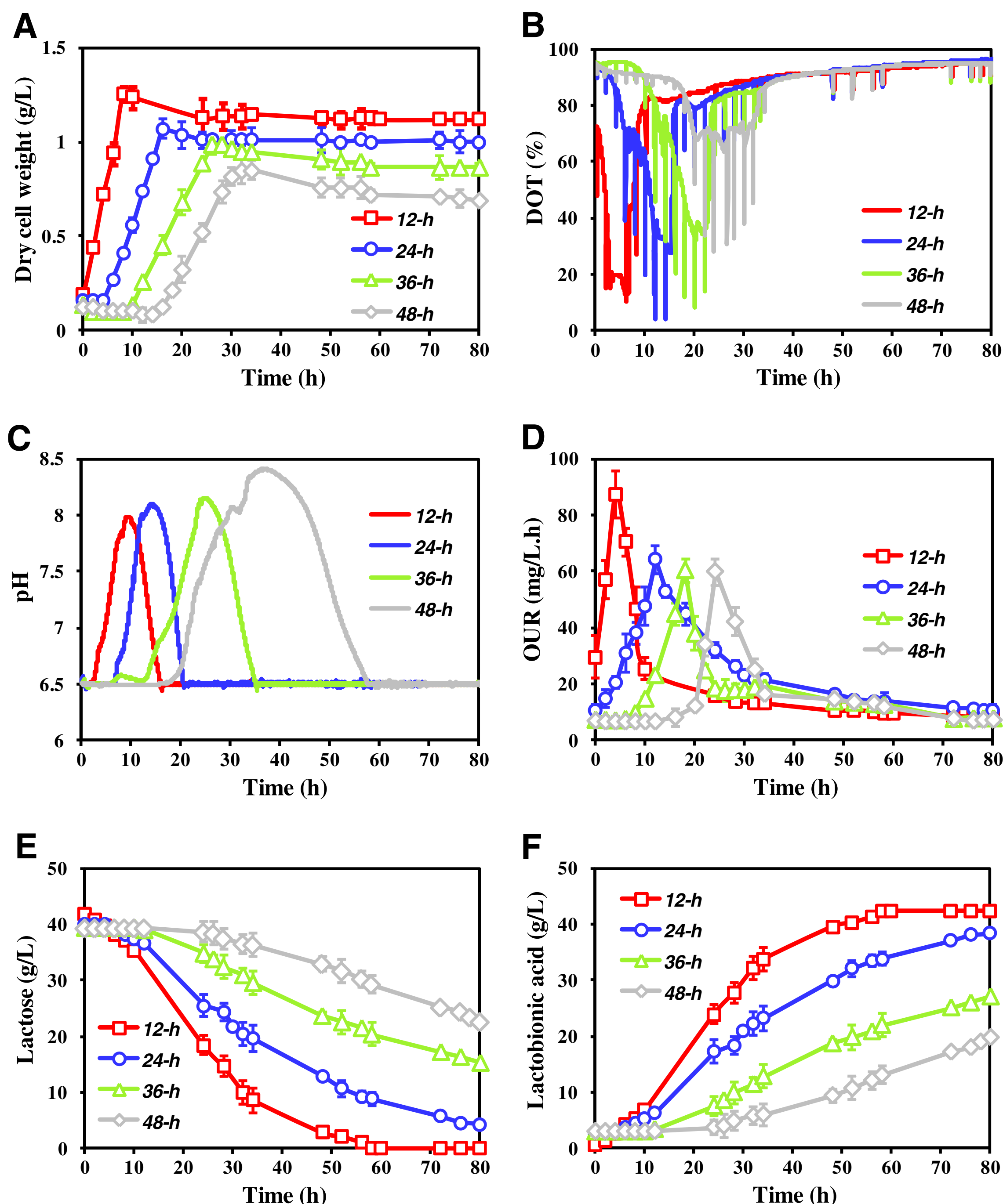

Fig. 6 Time-course profiles of cell growth (**a**), DOT (**b**), pH (**c**), OUR (**d**), lactose (**e**), and lactobionic acid (**f**) during pH shift bioreactor cultivations employing a seed culture age of 12, 24, 36, or 48 h

lactobionic acid production phase began at 38 h when employing a 48-h seed culture compared to 10 h when a 12-h seed culture was inoculated.

Dissolved oxygen profiles were strongly related to the growth phase, as can be appreciated in Fig. 6b. A pronounced depletion in dissolved oxygen concentration was found at the initial stage of the cell growth phase followed by a sudden rapid increase to saturated values, indicating the onset of the production phase. Furthermore, there was a substantial difference in the minimum DOT levels obtained in the fermentation broth. A minimum DOT level of 20 % was reached during growth phase employing a 12-h seed culture compared to the 70 % obtained when using a 48-h seed culture (Fig. 6b).

As regards pH profiles, fermentation broths suffered the usual sharp increase in pH values during the exponential

Table 1 Comparison of values obtained in pH shift bioreactor cultivations under the influence of different seed culture ages

Inoculum age (h)	Maximum dry cell weight (g/L)	Duration of the lag phase (h)	Onset of the production phase (h)	pH shift value	μ (h^{-1})	Volumetric productivity (g/L h)[a]	Culture time (h)	Yield (%)[b]
12	1.25±0.04	0	10	8.00±0.05	0.19	0.70±0.00	58	100±0
24	1.07±0.05	4	16	8.02±0.08	0.15	0.45±0.02	80	90±2
36	0.98±0.05	8	26	8.19±0.03	0.10	0.30±0.02	80	63±1
48	0.85±0.07	16	38	8.43±0.02	0.06	0.20±0.03	80	46±2

μ specific growth rate

[a] Data were calculated considering each culture time

[b] Yield was defined as the percentage of lactose converted into lactobionic acid after each culture time

growth phase (Alonso et al. 2011, 2012), as can be seen in Fig. 6c. The increase in seed culture age led to a progressive rise in pH shift values (8.00, 8.02, 8.19, and 8.43 for 12, 24, 36, and 48 h, respectively). The maximum oxygen demand by *P. taetrolens* cells coincided with the proliferation phase, as can be appreciated in Fig. 6d, whereas OUR values remained constant or slightly reduced at the onset of the production phase. The cultivation inoculated with the youngest seed culture (12 h) presented a maximum OUR value of 87.5 mg/L h at 4 h, compared to similar maximum OUR values of 64, 61, and 60 mg/L h obtained when employing seed culture ages of 24, 36, and 48 h, respectively.

As can be seen in Fig. 6f, an increase in seed culture age resulted in a marked decrease in the lactobionic acid titers produced by *P. taetrolens*. Both volumetric productivities and bioprocess yields were severely affected when employing prolonged time seed cultures (Fig. 7), being 1.55-, 2.3- and 3.5-fold (0.45, 0.30, and 0.2 g/L h for 24-, 36-, and 48-h seed cultures, respectively) lower than those obtained using the youngest seed culture (0.70 g/L h for a 12-h seed culture). These results provide evidence that senescent cultures employed as inocula exhibited a poor physiological status which impaired subsequent re-growth and lactobionic acid production. In accordance with the results obtained (Fig. 8), prolonged time seed cultures (36 and 48 h) were predominantly composed of dead cells, whereas healthy, metabolically active and damaged cells were the main cell subpopulations in the 12- (Fig. 5b) and 24-h (Fig. 8b) seed cultures, respectively. As Fig. 8a shows, cells from this latter culture showed a growth advantage over cells from 36- and 48-h seed cultures, seeing as they rapidly entered into a metabolically active status. Thus, once cells adapted their cellular machinery (identified by the disappearance of the lag phase), dead and damaged subpopulations were partially replaced by cells that remained metabolically active (Fig. 8b, c).

Thus, the use of an active healthy seed culture minimized the duration of the lag phase in the subsequent pH shift cultivation and also the timing thereof for switching from growth to product formation. Prolonged time cultures (longer than 12 h) employed as seed cultures displayed poor bioconversion efficiencies as well as poor fermentation performances (Fig. 7) due to the presence of damaged and dead cells in these cultures (Fig. 8).

Discussion

Identifying the complex population dynamics as well as the underlying physiology currently remains one of the major challenges for the industrial development of many bioprocesses in which microbial biomass would otherwise be approached as a whole steady-state bulk parameter, consequently leading to misinterpretations. In contrast, assessment

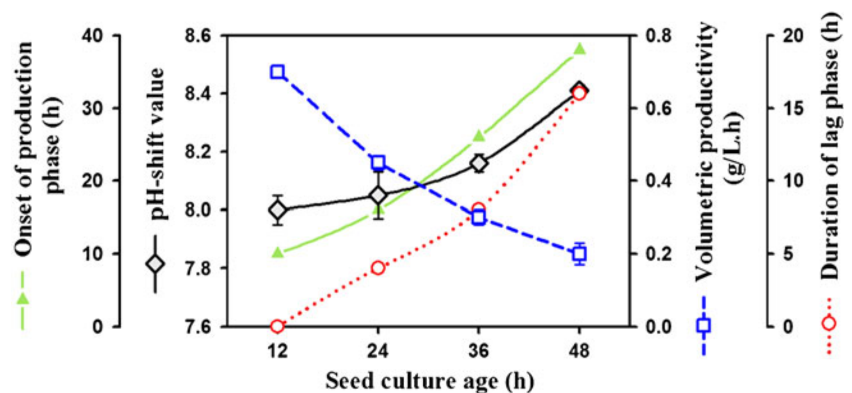

Fig. 7 Relationship between seed culture age and bioprocess parameters obtained during batch cultivations

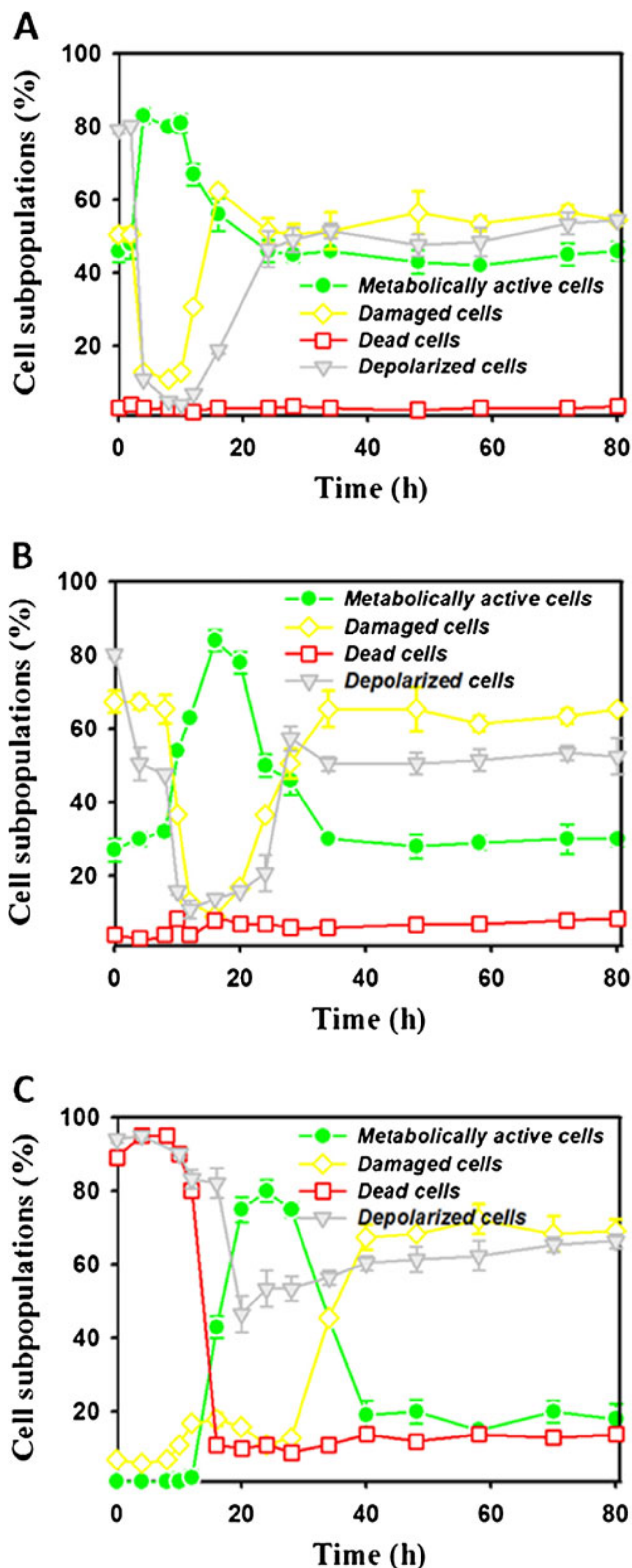

Fig. 8 Time-course profiles of cell subpopulations during pH shift bioreactor cultivations employing a seed culture age of 24 (**a**), 36 (**b**), or 48 h (**c**)

of the physiological state of cells by means of multiparameter flow cytometry can provide relevant information on the *health status* of cells as well as valuable guidelines on bioprocessing conditions for enhanced microbial performance (Hewitt and Nebe-von-Caron 2001; Lopes da Silva et al. 2009). In this study, flow cytometric assessment of *P. taetrolens* cells has revealed major functional and physiological changes during lactobionic acid production from whey, in addition to deciphering the influence of this physiological heterogeneity on culture performance.

Shake-flask cultivation showed that *P. taetrolens* cells suffered a progressive loss of metabolic activity and membrane integrity compared to bioreactor cultivations, in which the imposed pH shift bioprocessing conditions supported a robust physiological status (Figs. 2b and 5b). In shake-flask fermentation, the transition from the growth to the stationary growth phase was accompanied by an abrupt loss in cellular parameters. Thus, physiologically and functionally compromised cells were predominant during the later stages of shake-flask fermentation, suggesting that prolonged exposure to the acidic environment first led to a damaged state and then to cellular death (Figs. 2b and 3a). Cellular parameters such as membrane potential, membrane integrity, pump activity, or metabolic activity are identified as the main indicators of microbial viability (Hewitt and Nebe-von-Caron 2004; Hammes et al. 2011). In fact, cells with damaged or compromised membranes are not able to maintain or generate the electrochemical gradient which is involved in the generation of energy or nutrient transport (Díaz et al. 2010). In this regard, loss of membrane potential usually arises during the stationary phase due to higher stressing levels or deleterious bioprocessing conditions (David et al. 2011; Nielsen et al. 2009).

Undoubtedly, exposure to deleterious bioprocessing conditions involves major physiological responses from bacteria, as cellular homeostasis and the metabolic machinery of the cells are highly influenced by the surrounding microenvironment (Nicolau et al. 2010). The onset of the stationary phase in gram-negative bacteria also comprises major morphological changes through an alteration of their size and shape (Navarro et al. 2010). In fact, *P. taetrolens* cells became smaller and spherical at the late stationary phase compared to the rod-shaped form they showed at early stages of shake-flask cultivation (Fig. 3a). According to the data, the functional status of *P. taetrolens* was intimately linked to the extreme environment encountered during shake-flask cultivation (pH=3.35), seeing as cells could not maintain optimal homeostasis under prolonged exposure to acidic conditions (Fig. 2a, b). These severe conditions clearly led to *P. taetrolens* cells entering the death phase as well as the formation of cellular aggregates (Fig. 3a). Conversely, cells remained predominantly healthy and metabolically active (>96 %) in addition to displaying an intact depolarized membrane throughout pH shift bioreactor cultivation (Fig. 5b), highlighting the suitability of this

cultivation strategy. Accordingly, pH was controlled above 6.5 during the lactobionic acid production phase (Fig. 5a) in order to prevent the deleterious bioprocessing conditions encountered in shake-flask cultivation (Fig. 2a, b).

Physiological heterogeneity represents the main source of poor biological performance and yield in microbial cultures (Avery 2006; Müller et al. 2010). In accordance with the results, fermentation performance was strongly affected by the use of senescent seed cultures older than 12 h (Fig. 6), highlighting the importance of an appropriate time criteria for harvesting the seed culture. Accordingly, a seed culture of 12 h (mostly comprising healthy, metabolically active cells, as shown in Fig. 5b) may be established as the best physiological age in terms of biological performance (Fig. 6). Otherwise, the prevalence of sublethally injured or dead cells in the seed culture could impair bioconversion efficiency as well as the adaptive response by *P. taetrolens* cells once inoculated (Figs. 7 and 8).

Considered as a whole, this study has provided an approach for optimizing lactobionic acid production from whey based on the physiological status of *P. taetrolens*, deciphering for the first time the role played by this underlying physiology in fermentation performance and lactobionic acid productivity. Furthermore, the use of this information could be of considerable interest for the development and optimization of biotechnological systems targeting the microbial production of secondary metabolites.

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4.6. Influencia del pH en la producción de ácido lactobiónico

En este trabajo se ha buscado descifrar la influencia del pH operacional sobre la viabilidad celular de *P. taetrolens* y cómo la heterogeneidad fisiológica resultante está directamente imbricada en la producción de ácido lactobiónico. Se han empleado estrategias de control de pH no descritas hasta la fecha (estrategia *pH-shift* a diferentes valores, diferentes valores de pH inicial e incluso valores de pH estrictamente controlado) con el objetivo de evaluar la influencia de las mismas sobre la producción de ácido lactobiónico a partir de suero lácteo.

Tradicionalmente, se ha venido enfocando el estudio de todo bioproceso obviando la influencia del pH microambiental sobre la heterogeneidad fisiológica del sistema. En este trabajo, se ha optimizado un sistema fermentativo por primera vez en base a conocimientos fisiológicos aportados por la citometría de flujo multi-paramétrica. Así, una vez establecidas las condiciones óptimas de aireación/agitación y la edad del inóculo (véanse anteriores subapartados), se ha procedido a optimizar el valor óptimo de pH inicial, así como la estrategia de control de pH más adecuada en términos productivos o en base a consideraciones fisiológicas de *P. taetrolens*. Los resultados han revelado la indudable influencia tanto del pH inicial como de la estrategia de control de pH adoptada. Se ha perseguido en definitiva combinar ambos conocimientos con el fin de proponer la metodología más adecuada desde ambas perspectivas, llegando a señalar a un pH inicial de 6,5 y una estrategia de control estricto de pH (estrategia *pH-stat*) o bien de control en fase de producción (estrategia *pH-shift*) en 6,5 como las estrategias óptimas desde el punto de vista productivo.

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Selection method of pH conditions to establish *Pseudomonas taetrolens* physiological states and lactobionic acid production

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Abstract Microbial physiological responses resulting from inappropriate bioprocessing conditions may have a marked impact on process performance within any fermentation system. The influence of different pH-control strategies on physiological status, microbial growth and lactobionic acid production from whey by *Pseudomonas taetrolens* during bioreactor cultivations has been investigated for the first time in this work. Both cellular behaviour and bioconversion efficiency from *P. taetrolens* were found to be negatively influenced by pH-control modes carried out at values lower than 6.0 and higher than 7.0. Production schemes were also influenced by the operational pH employed, with asynchronous production from damaged and metabolically active subpopulations at pH values lower than 6.0. Moreover, *P. taetrolens* showed reduced cellular proliferation and a subsequent delay in the onset of the production phase under acidic conditions (pH<6.0). Unlike cultivations performed at 6.5, both pH-shift and pH-stat cultivation strategies performed at pH values lower than 6.0 resulted in decreased lactobionic acid production. Whereas the cellular response showed a stress-induced physiological response under acidic conditions, healthy functional cells were predominant at medium operational pH values (6.5–7.0). *P. taetrolens* thus displayed a robust physiological status at initial pH value of 6.5, resulting in an enhanced bioconversion yield and lactobionic acid productivity (7- and 4-fold higher compared to those attained at initial pH values of 4.5 and 5.0, respectively). These results have shown that pH-control modes strongly affected both the physiological response of cells and the biological performance of *P. taetrolens*, providing key information for bio-production of lactobionic acid on an industrial scale.

Keywords Flow cytometry · pH-control strategy · Lactobionic acid · Bioprocessing conditions · *Pseudomonas taetrolens* · Physiological status

Introduction

Microbial stress responses can frequently be found associated with inappropriate bioprocessing conditions in many industrially relevant bioprocesses. Microorganisms constantly face environmental fluctuations which may induce the development of complex phenotypes for an enhanced tolerance against potentially adverse conditions (Hewitt et al. 2007; Nicolau et al. 2010; Onyeaka et al. 2003). As a result, clonal bacterial populations may develop a stress-induced physiological response which can lead to a heterogeneous culture comprising cells with different physiological states (Lidstrom and Konopka 2010; Müller et al. 2010). Such knowledge regarding cell-to-cell heterogeneity within a bioprocess can be suitably addressed with multi-parameter flow cytometry (Díaz et al. 2010), which enables the detection and monitoring of changes in cellular constituents and properties or the overall physiological status of microorganisms otherwise unidentified by conventional culture-based approaches (Hewitt and Nebe-von-Caron 2001, 2004). Flow cytometry has therefore become an outstanding tool for bioprocess monitoring and optimization in view of the fact that it can provide valuable guidelines on the most suitable bioprocessing conditions (David et al. 2011; Delvigne et al. 2011; Silva et al. 2011). Information on functional status and physiological robustness accordingly enables the successful implementation of non-deleterious fermentation

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strategies, essentially resulting in improved microbial fitness and biological performance (Lopes da Silva et al. 2009).

Undoubtedly, microenvironmental pH constitutes a critical bioprocessing parameter that exerts a major influence on microbial physiology. Inappropriate bioprocessing conditions may thus involve the development of asynchronous cell subpopulations comprising metabolite producing and non-producing cells, which has a profound impact on fermentation performance (Nicolau et al. 2010; Want et al. 2009). Although numerous approaches have focused on deciphering the influence of pH in submerged liquid fermentations, the physiological response and segregation of microbial subpopulations due to inappropriate operational pH conditions have scarcely been evaluated so far.

Determining the deleterious impact of such bioprocessing conditions could be of substantial importance for the development of incipient, industrially relevant bioprocesses targeting novel bio-products such as lactobionic acid. The cosmetics and pharmaceutical industries have recently shown considerable interest in lactobionic acid as the key active component of novel therapeutic formulations (Green et al. 2009) or as a chemical platform for the synthesis of bioactive molecules (Ortial et al. 2006). Lactobionic acid is a high value-added polyhydroxy acid which additionally presents a growing niche market with applications in the fields of foodstuffs (Gerling 1998) and medicine (Southard and Belzer 1995; Peng et al. 2007). To date, lactobionic acid is industrially produced by chemical synthesis in an energy-intensive process using costly metal catalysts (Kuusisto et al. 2007). However, bio-based lactobionic acid production from an inexpensive feedstock such as cheese whey can be efficiently accomplished through a biotechnological process (Alonso et al. 2011, 2012a) in which both fermentation performance and productivity are strongly reliant on the physiological status displayed by *Pseudomonas taetrolens* (Alonso et al. 2012b). Consequently, deciphering the detrimental influence of the operational pH mode on the cellular behaviour of *P. taetrolens* constitutes a key issue in order to establish the optimal bioprocessing conditions for enhanced fermentation performance. Identifying both the cellular behaviour and underlying physiology involved constitutes an essential step for the industrial development of any bioprocess in which the microbial biomass would be otherwise approached erroneously as a whole steady-state bulk parameter (Hewitt and Nebe-von-Caron 2004).

Hence, the aim of the present study was to assess the impact of different pH-control strategies (uncontrolled-pH, pH-stat, pH-shift and initial pH) on lactobionic acid production as well as on the physiological status of *P. taetrolens*. To this end, the physiological heterogeneity of *P. taetrolens* during batch bioreactor cultivations was monitored through multi-parameter flow cytometry in order to determine the deleterious influence of these bioprocessing conditions on fermentation performance. The information obtained from cellular parameters such as membrane polarization, membrane integrity and metabolic activity may thus enable the implementation of novel process strategies aimed at preventing any potential detrimental influence.

Materials and methods

Microorganism

P. taetrolens LMG 2336, obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium), was maintained frozen (in 40 % [v/v] glycerol at −20 °C). This strain was subsequently subcultured on Nutrient Broth (NB; containing 1 g/l meat extract, 2 g/l yeast extract, 5 g/l peptone and 5 g/l NaCl) agar plates, incubated for 48 h at 30 °C and then preserved at 4 °C.

Inoculum preparation

A loopful of *P. taetrolens* from a fresh NB agar plate was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of NB medium. This flask was incubated on an orbital shaker (Infors HT, model Flyer Aerotron, Switzerland) at 250 rpm and 30 °C for 10 h. Actively growing cells from this culture were then employed as inoculum for the bioreactor seed cultures.

Sweet whey preparation

Sweet cheese whey (provided by ILAS S.A., Asturias, Spain) was 1-fold diluted with distilled water (1:1) and adjusted to pH 6.5 (by adding 6 M NaOH) prior to sterilization using a tangential microfiltration device equipped with a 0.22 μm pore size PVDF membrane-cassette (Millipore, Massachusetts, USA).

Bioreactor cultivations

Batch cultivations were performed in a 2-l bioreactor (BioFlo 110; New Brunswick Scientific, New Jersey, USA) with a working volume of 1 l. Bioreactor experiments were conducted at 30 °C with an inoculation level of 10 % (v/v) and an agitation rate of 350 rpm. Besides, the bioreactor was aerated (1.0 vvm) via a ring sparger located at the bottom of the vessel. Excessive foam formation was prevented by automatic addition of diluted (1:10) Y-30 emulsion (Sigma-Aldrich, Steinheim, Germany). The bioreactor was equipped with a pH meter (Mettler Toledo, Switzerland) and a polarographic dissolved oxygen electrode (InPro 6830; Mettler Toledo) for continuous online monitoring of pH and dissolved oxygen tension (DOT) values, respectively. The seed culture was obtained by

harvesting biomass (after centrifugation at 11,000×*g* for 10 min) from a NB shake-flask culture and re-suspending cells in a 500-ml Erlenmeyer flask containing 100 ml sweet whey. Subsequently, these cultures were grown at 250 rpm in an orbital shaker at 30 °C for 12 h and then employed as seed culture in the bioreactor at 10 % (v/v) containing sweet whey. These prior conditions were applied to all cultivations unless otherwise specified. Cultivations were carried out in duplicate as independent experiments.

Uncontrolled-pH bioreactor cultivation

In order to gain insight into how the system and bioprocess parameters are affected by the lack of pH-control, bioreactor cultivation was performed under uncontrolled-pH conditions. The pH was accordingly left to vary freely after adjusting the initial pH to 6.5 (by adding 3 M NaOH).

Influence of initial pH on bioreactor cultivations

The influence of different initial pH values (4.5, 5.0, 6.0, 6.5, 7.0 and 7.5) on bioprocess parameters combined with a pH-shift control strategy was evaluated. A two-stage pH-shift bioconversion strategy was thus adopted in bioreactor cultivations as previously detailed (Alonso et al. 2011). After adjusting the initial pH to its corresponding value, the pH was left uncontrolled during the growth phase and subsequently maintained at 6.5 during the production phase by means of computer-controlled peristaltic pumps via automatic addition of 3 M NaOH.

Bioreactor cultivations under different pH-shift control values

The aforementioned pH-control strategy was adopted at different pH-shift values after the onset of the production phase. The pH was therefore left uncontrolled above 6.5 during the growth phase and then maintained at different pH values (4.5, 5.0, 6.0, 6.5, 7.0 and 7.5) during the production phase through automatic addition of 3 M NaOH.

Bioreactor cultivations under different pH-stat conditions

In order to examine the influence of different pH-stat values on the bioprocess parameters, bioreactor cultivations were carried out with continuous pH control at 4.5, 5.0, 6.0, 6.5, 7.0 and 7.5 through automatic addition of either 3 M NaOH or 3 M H_2SO_4. By applying this bioconversion strategy, the culture broth basicity associated with the cellular growth phase is avoided (Alonso et al. 2011).

Staining procedures

Samples from cultures were harvested by centrifugation at 16,000×*g* for 5 min. Before staining, cells were washed twice in phosphate-buffered saline (PBS; pH 7.4, sterile and filtered at 0.22 μm) and were then held in the "hot spot" of a sonication bath for 2 s to prevent bacterial aggregation before flow cytometric analysis (Hewitt and Nebe-von-Caron 2004). Propidum iodide (PI; Invitrogen), bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol, $DiBAC_4(3)$; Invitrogen) and carboxyfluorescein diacetate (cFDA; Invitrogen) were used as fluorescent dyes in a double dual-staining procedure ($DiBAC_4(3)$/PI and cFDA/PI) in order to evaluate cell physiological status (metabolic activity, membrane integrity and membrane polarization were evaluated through cFDA, PI and $DiBAC_4(3)$ staining, respectively). Staining procedures were performed as previously reported by Alonso et al. (2012b).

Multi-parameter flow cytometry

Flow cytometry measurements were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with a 488 and 633 nm excitation light source from an argon ion laser. Green fluorescence from samples (corresponding to $DiBAC_4(3)$ and cFDA-stained cells) was collected on the FL1 channel (530 nm), whereas PI fluorescence was registered on the FL3 channel (610 nm). Each analysis was performed in duplicate at a low flow rate setting (4,000 events/s). Data acquisition was carried out using Cytomics RXP software (Beckman Coulter). Gates and quadrants were established according to staining controls. For $DiBAC_4(3)$/PI and cFDA/PI dual-parameter flow cytometric analysis, data collected from 150,000 and 100,000 events, respectively, were analyzed using Summit v4.3 software (DakoCytomation, Colorado, USA).

Analytical methods

Bacterial growth was measured spectrophotometrically as the optical density at 600 nm (Shimazdu, UV 1203 model) after centrifugation of culture samples at 16,000×*g* for 5 min. Optical density data was converted to cell dry weight (expressed in grams per litre) using the corresponding previously obtained calibration curve. The lactobionic acid and lactose content of cell-free culture samples was measured by high performance liquid chromatography as reported previously by Alonso et al. (2011).

Determination of the oxygen uptake rate

The oxygen uptake rate (OUR) from bioreactor cultivations was determined via the dynamic method as previously

reported (Alonso et al. 2012a). The specific consumption rate of oxygen (qO_2) was calculated by dividing the OUR value by the biomass concentration at each sampling point.

Results

Uncontrolled-pH bioreactor cultivation

Figure 1 shows the influence of uncontrolled-pH conditions on bioprocess parameters during bioreactor fermentation. As can be seen, the onset of the stationary growth phase featured the return of the DOT to values near to the air saturation level concomitant to an initial sharp decrease (from 10 to 24 h) and subsequent progressive decay in pH (from 24 h onwards). The pattern of lactose oxidative bioconversion from whey by *P. taetrolens* cells showed the typical non-growth associated production, as previously reported (Alonso et al. 2011). However, the synthesis of lactobionic acid ceased after 34 h with a production rate of 0.35 g/lh, leaving 14 g/l of residual lactose (Fig. 1a). Despite the fully aerobic conditions, cells exhibited a low specific oxygen consumption rate (around 2 mg/gh) throughout the lactobionic acid formation phase which also featured the predominance of damaged cells. Thus, *P. taetrolens* cells were predominantly (>90 %) healthy and metabolically active during the growth phase, whereas damaged cells constituted the main cell subpopulation (>90 %) from 34 h onwards (Fig. 1b). Additionally, the transition from the growth to the stationary phase featured the prevalence of depolarized cells (>85 % from 24 h onwards), suggesting that the onset of lactobionic acid synthesis was intimately linked to changes in the cytoplasmic membrane polarization of *P. taetrolens* cells.

Effect of initial pH on bioprocess parameters

Figure 2 shows the impact of initial pH values on bioprocess parameters during bioreactor cultivations performed with a pH-shift strategy at 6.5. This two-stage pH-control strategy was devised to avoid the negative influence exerted by the uncontrolled-pH conditions on lactose oxidative bioconversion (Fig. 1a). Whereas maximum biomass values were clearly impaired by initial pH values lower than 6.0, cell growth was stimulated under cultivations performed at initial pH higher than 7.0 (Fig. 2a). Moreover, initial pH values ranging from 6.0 to 6.5 contributed to enhanced cellular proliferation, resulting in specific growth rates of 0.18 and 0.19 h^{-1} for an initial pH of 6.0 and 6.5, respectively (Table 1). As shown in Fig. 2a, cultivation at an initial pH of 4.5 showed a lag phase of 8 h, thereby delaying the onset of the production phase up to 34 h. The lag phase suggests poor physiological robustness and further biological performance since its presence is a signal of the lack of an adaptive response from *P. taetrolens* cells. The DOT profiles (Fig. 2b) showed that *P. taetrolens* cells were barely consuming oxygen under initial pH values of 4.5 and 5.0, since DOT values were near the saturation level. This was consistent with the oxygen consumption rate from *P. taetrolens* cells under these circumstances, given that the OUR values decreased progressively as fermentation proceeded (Fig. 2d).

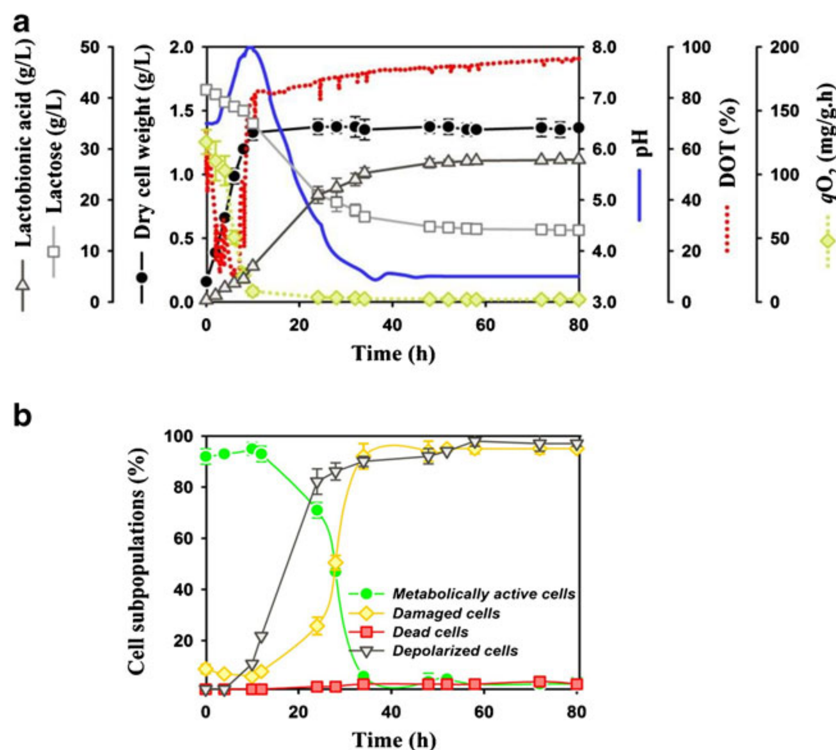

Fig. 1 Time-course profile of bioprocess parameters obtained during uncontrolled-pH bioreactor cultivation of *P. taetrolens* (**a**). Percentages of *P. taetrolens* cell subpopulations throughout bioreactor fermentation (**b**)

Fig. 2 Time-course profiles of cell growth (**a**), DOT (**b**), pH (**c**), OUR (**d**), lactose (**e**) and lactobionic acid (f) during pH-shift bioreactor cultivations at different initial pH values

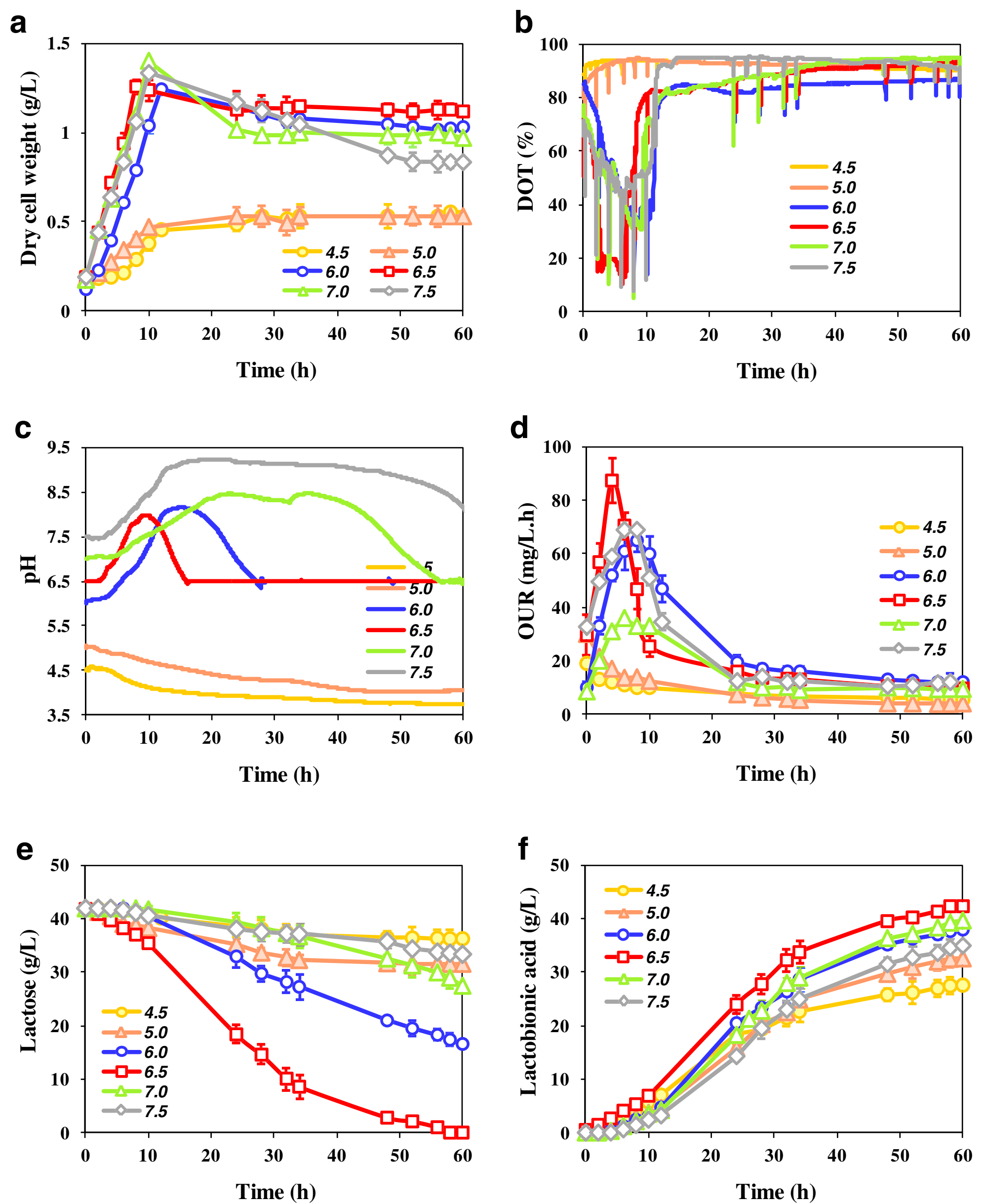

Table 1 Comparison of values obtained in bioreactor cultivations under the influence of different initial pH values

Initial pH	Maximum dry cell weight (g/l)	Duration of the lag phase (h)	Onset of the production phase (h)	pH-shift value	μ (h^{-1})	Volumetric productivity (g/lh)	Yield (%)[a]
4.5	0.55±0.04	8	34	–	0.02	0.1±0.00	14±1
5.0	0.55±0.02	2	28	–	0.10	0.18±0.02	25±1
6.0	1.24±0.03	0	12	8.09±0.08	0.18	0.42±0.04	58±2
6.5	1.25±0.04	0	10	8.00±0.05	0.19	0.70±0.00	100±0
7.0	1.40±0.03	0	34	8.47±0.03	0.15	0.22±0.02	31±3
7.5	1.33±0.06	0	48	9.22±0.02	0.13	0.15±0.02	20±2

– absence of a pH-shift value; μ=specific growth rate

[a] Yield was defined as the percentage of lactose converted into lactobionic acid after 58 h

Lactobionic acid production schemes were highly influenced by the initial pH value, as shown in Fig. 2f. Initial pH values lower than 5.5 induced the onset of lactobionic acid formation without the end of the cellular growth phase, whereas initial pH values above 5.5 induced the simultaneous formation of lactobionic acid with cellular proliferation. As a result, both fermentation efficiency and lactobionic acid yield were clearly reduced under pH values higher than 7.0, resulting in a 4-fold lower yield (25 %) at an initial pH of 5.0 compared to that obtained at 6.5 (100 % yield). In terms of volumetric productivity, bioreactor cultivation at an initial pH of 6.5 (0.7 g/lh) resulted in a 7- and 4.7-fold higher value compared to that achieved at 4.5 (0.1 g/lh) and 7.5 (0.15 g/lh), respectively. The presence of lag phases at initial pH values lower than 6.0 (being 8 and 2 h for 4.5 and 5.0, respectively) clearly delayed the onset of the production phase, which was prolonged up to 34 and 28 h at initial pH values of 4.5 and 5.0, respectively (Table 1). Regardless of the absence of lag phases at initial pH values higher than 6.5, the delay in the onset of the lactobionic acid production phase was longer than at acidic values (i.e., 4.5 or 5.0) (Fig. 2e and f), which was indicative of overflow metabolism under these circumstances.

Regarding physiological status, reduced initial pH values severely affected the physiology of *P. taetrolens* cells, as indicated by a decrease in the healthy, metabolically active subpopulation (Fig. 3). At an initial pH of 4.5, metabolic activity decreased with two distinct subpopulations emerging (damaged and dead), thereby leading to decreased biological performance (Fig. 3a). This progressive increase in the damaged subpopulation thus involved asynchronous lactobionic acid production comprising producing and non-producing cells. In contrast, cells were predominantly healthy and metabolically active (>90 %) throughout the cultivation at an initial pH of 6.5 and 7.5, although the onset of the lactobionic acid production phase involved a depolarization of the cellular membrane (Fig. 3c and d).

Influence of pH-shift bioconversion values

In order to elucidate the role of pH-shift control on lactobionic acid production, bioreactor cultivations were carried out under a pH-shift bioconversion strategy (Fig. 4). Obviously, cellular proliferation patterns were similar during the growth phase (Fig. 4a), although cultivations performed under acidic conditions (pH-shift=4.5, 5.0) supported higher cell densities during the stationary growth phase compared to those performed at higher pH-shift values. Upon reaching their maximum biomass at 10 h, the transition from the growth to the stationary growth phase was accompanied by an abrupt increase in DOT values (Fig. 4b). As regards microbial oxygen requirements and consumption, all OUR patterns peaked and coincided at 8 h during the growth phase, as shown in Fig. 4d. The onset of the production phase featured a gradual decrease in the OUR, suggesting that the maximum catabolic activity of the cells was reached during the growth phase, subsequently remaining constant in the late stage of fermentation.

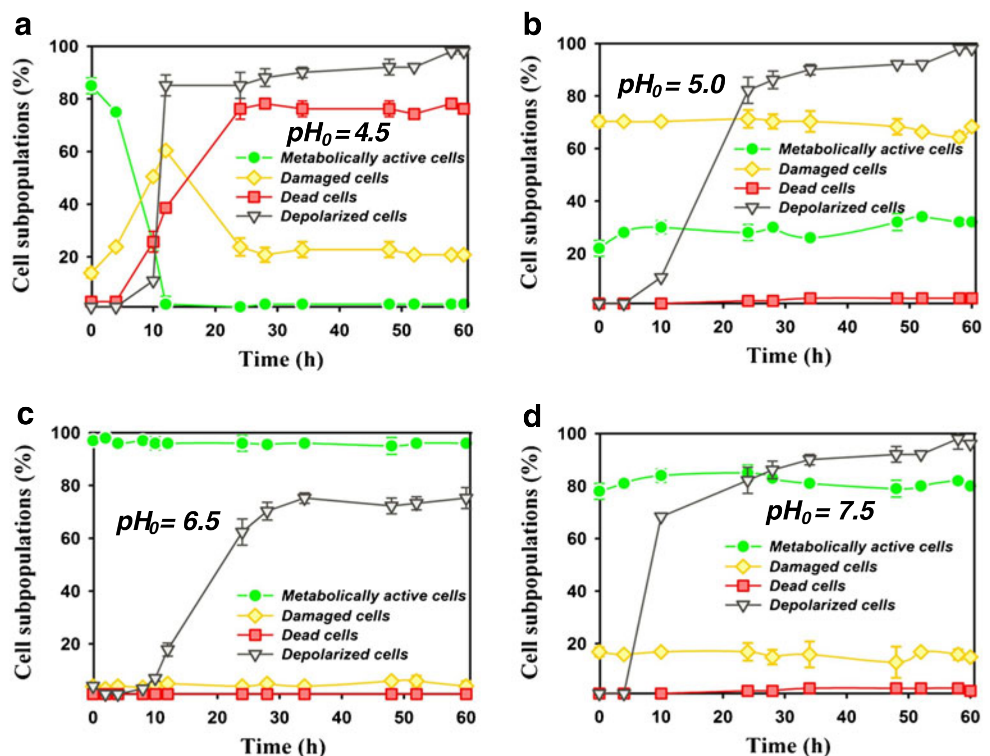

Fig. 3 Time-course profiles of cell subpopulations during pH-shift bioreactor cultivations performed at an initial pH of 4.5 (**a**), 5.0 (**b**), 6.5 (**c**) or 7.5 (**d**)

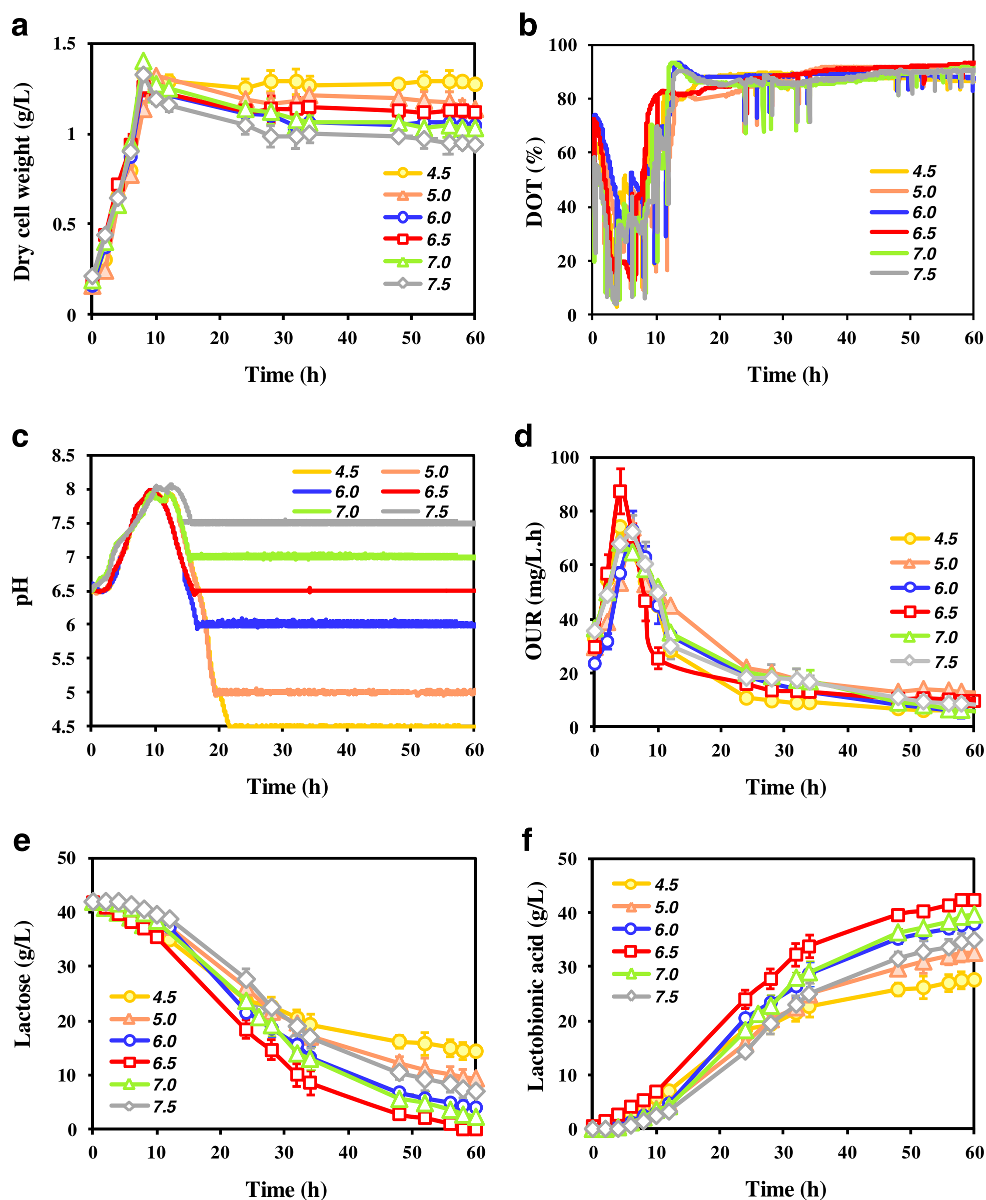

Fig. 4 Time-course profiles of cell growth (**a**), DOT (**b**), pH (**c**), OUR (**d**), lactose (**e**) and lactobionic acid (**f**) during bioreactor cultivations carried out at a pH-shift bioconversion value of 4.5, 5.0, 6.0, 6.5, 7.0 or 7.5

As can be seen in Fig. 4f, lactobionic acid decreased markedly not only with the increase in pH-shift values higher than 7.0, but also with values lower than 6.0. In fact, complete lactose bioconversion was achieved after 58 h at a pH-shift value of 6.5 compared to a 65 % process yield obtained at a pH-shift value of 4.5 (Table 2). Accordingly, the optimal pH-shift value for complete lactose bioconversion ranged from 6.5 to 7.0, as can be seen in Fig. 4e.

In accordance with the data shown in Fig. 5, both cultivations under a pH-shift bioconversion at 6.5 and 7.5 displayed a robust physiological status throughout the course of the fermentation. Cells exhibited high metabolic activity and retained their membrane integrity even at late stages of cultivation. Analysis of the physiological status of the cells revealed that 92 % of these cells remained healthy, metabolically active and polarized during the cell proliferation phase (Fig. 5c and d). Upon shifting to the production phase, no significant changes were found in terms of metabolic activity (around 92 % of the cells remaining active). In contrast, two phases were clearly observed at cultivations performed with a pH-shift strategy at 4.5 and 5.0: an active proliferation phase, in which cells were metabolically active and polarized, followed by the onset of the production phase, in which a marked decrease in the polarization level was detected (Fig. 5a and b). As these cultivations have revealed, metabolically active cells have been shown to be more effective towards lactobionic acid production compared to cells from pH-shift bioconversion strategies performed at 4.5 and 5.0, in which asynchronous production arose due to the presence of damaged cells (Fig. 5a and b). Therefore, responses of *P. taetrolens* cells to an acidic environment during the lactobionic acid production phase

Table 2 Comparison of values obtained in pH-shift bioreactor cultivations under different pH-shift values

pH-shift strategy	Dry cell weight (g/l)[a]	Onset of the production phase (h)	Volumetric productivity (g/lh)	Yield (%)[b]
4.5	1.30±0.00	10	0.47±0.00	65±0
5.0	1.15±0.02	10	0.56±0.00	77±0
6.0	1.11±0.08	10	0.65±0.04	90±2
6.5	1.13±0.04	10	0.70±0.00	100±0
7.0	1.04±0.05	10	0.68±0.02	94±2
7.5	0.95±0.06	10	0.59±0.02	82±2

μ=specific growth rate

[a] Maximum dry cell weight reached at the production phase

[b] Yield was defined as the percentage of lactose converted into lactobionic acid after 58 h

suggested that healthy, metabolically active cells assumed a damaged status, thereby displaying poor bioconversion performance. However, these pH-shift bioconversion conditions exerted lesser deleterious physiological effects than cultivation performed at different initial pH values, suggesting that changes in the microenvironmental pH were less detrimental during the lactobionic acid production phase.

pH-stat cultivations at different values

The potentially deleterious influence of inappropriate pH-stat conditions on both bioconversion performance and bioprocess parameters is shown in Fig. 6. As can be appreciated in Figs. 6 and 7, both bioprocess performance and physiological status were strongly dependent on the operational pH applied. The continuous pH-control strategy showed a positive influence on lactobionic acid titer and volumetric productivity (Table 3) compared to experiments carried out at different initial pH values (Table 1).

pH-stat cultivation strategies at medium pH values (6.0–6.5) contributed to enhanced cell growth of *P. taetrolens*, the highest maximum biomass concentration (1.36 g/l) being obtained at 6.5. Results from specific growth rates also revealed that pH-stat strategies ranging from 6.0 to 6.5 stimulated cell growth performance. The specific growth rate thus decreased on increasing the operational pH higher than 6.5, being 0.19 and 0.17 h^{-1} for pH-stat strategies at 7.0 and 7.5, respectively (Fig. 6a).

Dissolved oxygen profiles were strongly related to the growth phase, a sharp depletion in the dissolved concentration being found at the initial stage of the cell growth phase followed by a sudden rapid increase to saturated values (Fig. 6b). However, it was observed that the dissolved oxygen profile remained unchanged at saturation values

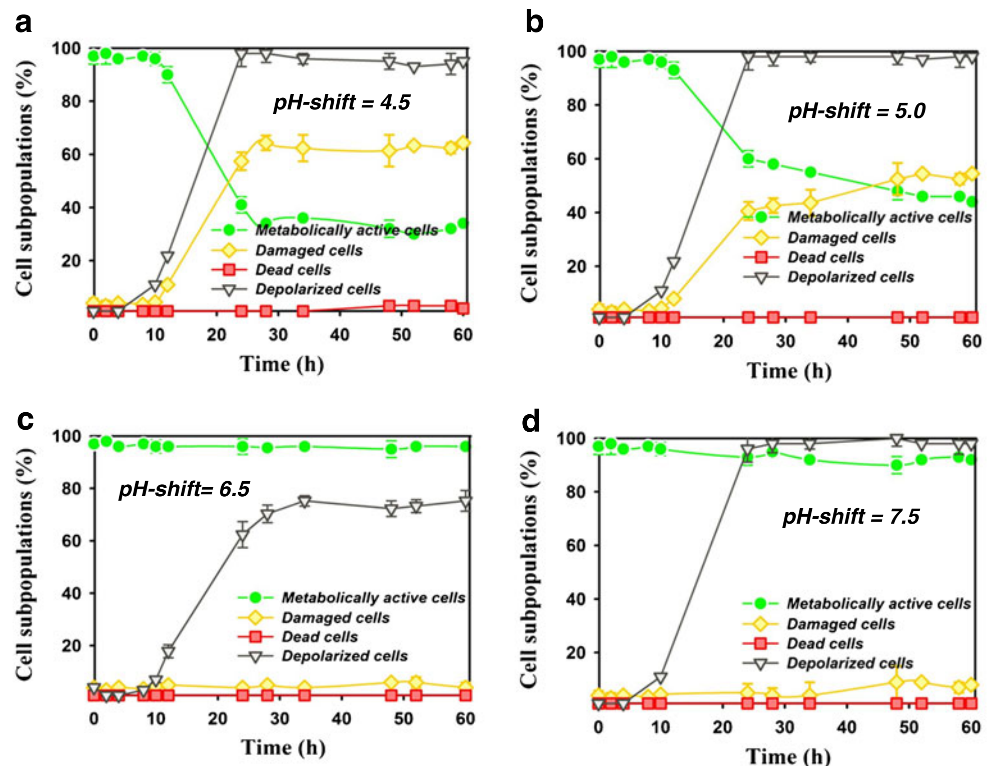

Fig. 5 Percentages of cell subpopulations during bioreactor cultivations carried out at a pH-shift bioconversion value of 4.5 (**a**), 5.0 (**b**), 6.5 (**c**) or 7.5 (**d**)

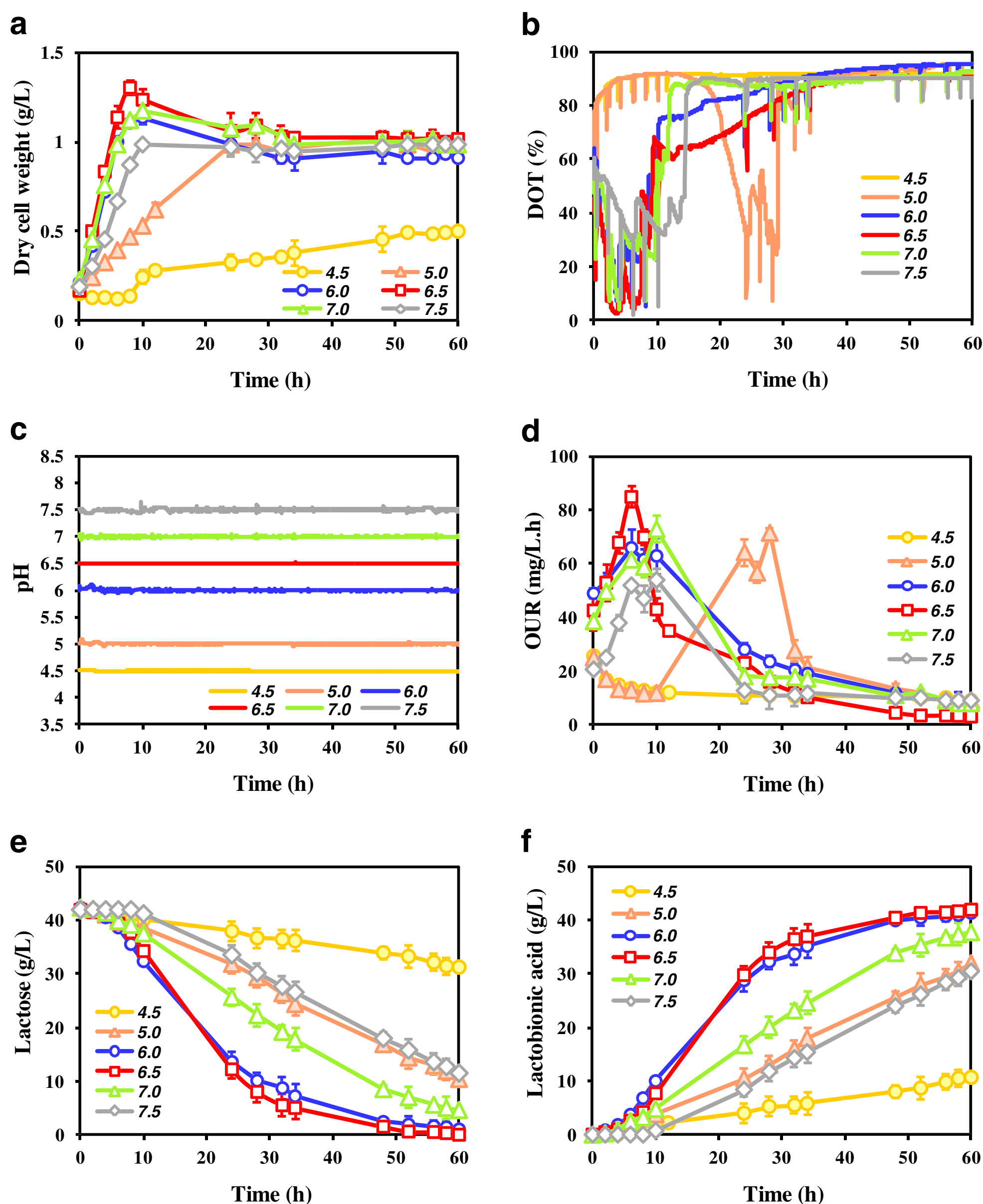

Fig. 6 Influence of the pH-stat strategy on cell growth (**a**), DOT (**b**), pH (**c**), OUR (**d**), lactose (**e**) and lactobionic acid (**d**) during bioreactor cultivations

throughout the cultivation performed under a pH-stat strategy of 4.5, thereby illustrating poor cellular proliferation under these conditions. Moreover, the temporal distribution of the maximum OUR values (with the exception of that from pH-stat cultivation at 4.5, which peaked at 28 h) took place at 6–10 h, coinciding with the end of the growth phase (Fig. 6d). As regards fermentation performance, both volumetric productivities and bioprocess yields were severely affected by the pH-stat cultivation strategy employed (Fig. 6e and f), being 1.4- and 4-fold lower (0.50 and 0.18 g/lh for pH-stat strategies at 7.5 and 4.5) than those obtained under a pH-stat strategy at 6.5 (0.70 g/lh). These results provide evidence that cultivations performed under pH-stat strategies ranging from 4.5 to 6.0 and from 7.0 to 7.5 exhibited a poor physiological status (Fig. 7) which impaired fermentation efficiency and therefore lactobionic acid production.

During pH-stat bioreactor cultivations at 6.5 and 7.5, cells remained predominantly healthy and functional throughout cultivation, as can be observed in Fig. 7c and d. In contrast, cultivation under pH-stat conditions lower than 6.0 showed an increase in the damaged cell subpopulation, leading to a stress-induced physiological response by *P. taetrolens* cells. Therefore, when the pH was strictly controlled at 4.5 and 5.0, both fermentation performance and lactobionic acid productivity were impaired due to the presence of non-producing lactobionic acid cells (Fig. 7a and b).

Interestingly, results have shown that maximum lactobionic acid titers were obtained at medium pH values (6.5–7.0), which differs substantially to the findings of

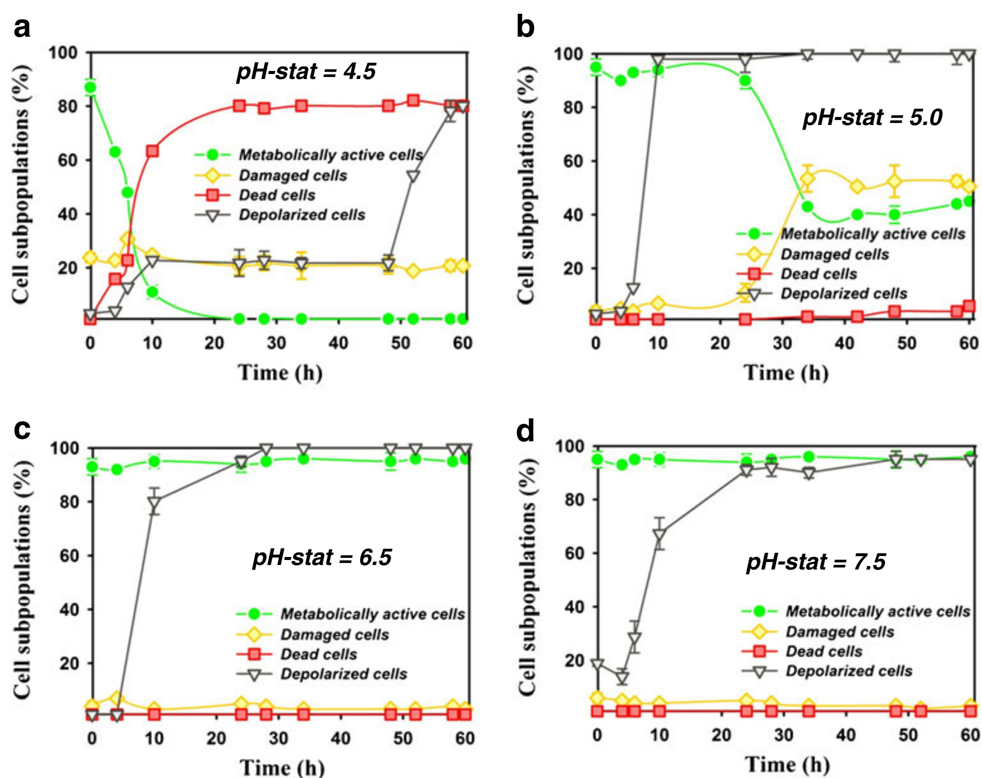

Fig. 7 Time-course profiles of cell subpopulations during pH-stat bioreactor cultivations performed at 4.5 (**a**), 5.0 (**b**), 6.5 (**c**) or 7.5 (**d**)

Nishizuka and Hayaishi (1962), who established 5.6 as the optimal pH value for the highest enzymatic lactose oxidase activity. In contrast to the present study, their experiments were performed under cell-free conditions, specifically with the purified membrane-located dehydrogenase system from *P. taetrolens* which mediates lactose oxidation and subsequent hydrolysis by lactonase to yield lactobionic acid.

Discussion

Environmental perturbations within bioprocesses often play a major role in the cellular homeostasis and metabolic machinery of microorganisms, therefore affecting their overall physiology (Baatout et al. 2007; Nicolau et al. 2010). The functional status of microorganisms may thus be strongly affected by the bioprocessing conditions encountered in a fermentation system. Inappropriate bioprocessing conditions could hence lead to major physiological responses with a marked impact on fermentation performance (Onyeaka et al. 2003; Want et al. 2009). In fact, stressful bioprocess conditions such as nutrient starvation, over-nutrient supply or hydrodynamic conditions may induce a stress-induced physiological response (Delvigne et al. 2011; Lopes da Silva et al. 2005; Silva et al. 2011) in which the membrane integrity or the metabolic activity of microorganisms is lost (Hewitt and Nebe-von-Caron 2004). Unlike healthy functional cells, microorganisms with compromised cytoplasmic membranes are not able to maintain or generate an electrochemical gradient and can hence neither create membrane potential nor carry on metabolic activity (David et al. 2011; Díaz et al. 2010). The present study has revealed for the first time how the cell growth, lactobionic acid production and fermentation efficiency of *P. taetrolens* have been markedly affected by the pH-control strategy adopted.

Table 3 Comparison of values obtained in bioreactor cultivations under different pH-stat values

pH-stat strategy	Maximum dry cell weight (g/l)	Duration of the lag phase (h)	Onset of the production phase (h)	μ (h^{-1})	Volumetric productivity (g/lh)[a]	Yield (%)[b]
4.5	0.53±0.03	8	34	0.01	0.18±0.00	25±0
5.0	1.01±0.05	0	24	0.08	0.52±0.02	72±2
6.0	1.13±0.08	0	10	0.20	0.69±0.00	97±0
6.5	1.30±0.03	0	10	0.21	0.70±0.00	100±0
7.0	1.17±0.04	0	10	0.19	0.64±0.02	88±2
7.5	0.98±0.06	0	24	0.17	0.51±0.02	70±2

μ=specific growth rate

[b]Yield was defined as the percentage of lactose converted into lactobionic acid after 58 h

Specifically, this identified lack of biological performance from *P. taetrolens* cells has been ascribed to major physiological responses due to deleterious pH conditions.

Despite the acidic conditions (pH=3.5) reached in uncontrolled-pH bioreactor cultivation (Fig. 1), cells remained damaged during the stationary growth phase, unlike in shake-flask fermentation, where prolonged exposure to an acidic as well as an oxygen-limited environment led to a stress-induced physiological response by *P. taetrolens* cells (Alonso et al. 2012b). Analysis of the pH-dependent physiological response has provided essential information on the bioconversion efficiency exhibited by *P. taetrolens* cells. Undoubtedly, microorganisms submitted to stressful pH conditions may undergo disruption of cellular homeostasis and functions, which can concomitantly lead to major physiological changes (Baatout et al. 2007). Unlike fully functional cells, sub-lethally injured and dead cells suffer from irreversible structural changes associated with the collapse of cellular functions such as the proton motive force (Hewitt and Nebe-von-Caron 2004). In fact, prolonged exposure to an acidic environment may involve the development of a reduced microbial phenotype or a lower degree of physiological robustness (Papadimitriou et al. 2007), which has a profound impact on biological performance and product metabolite yield.

Results have showed that optimization of the pH-control strategy led to enhanced lactobionic acid yields as well as improved fermentation performances (Tables 1, 2 and 3). Undoubtedly, the poor biological performance from *P. taetrolens* cells was attributable to the underlying physiological heterogeneity displayed by cultivations submitted to deleterious microenvironmental pH conditions (Figs. 3, 5 and 7). Whereas pH-control strategies (both pH-shift and pH-stat control) at 6.5 provided support for a robust physiological status, cultivations performed at lower values (i.e., 4.5 or 5.0) exhibited a complex physiological heterogeneity (mainly comprised by damaged and dead cells) that translated into reduced lactobionic acid productivity (4-fold lower under pH-stat conditions at 4.5 compared to that obtained at 6.5). These results demonstrate that an appropriate pH-control strategy may enhance microbial culture performance and hence increase the lactobionic acid titer. Lactobionic acid production phase may also be influenced by the cellular phenotypic diversity on the expression of lactose dehydrogenase system. However, experiments carried out at different pH-shift values (Fig. 4) suggested that differences at this molecular level could play a minor role in the bioconversion performance, assuming similar gene expression levels in the cells submitted to the same pH conditions during growth phase. Undoubtedly, the induced-stress physiological responses may impact on the bioconversion ability of *P. taetrolens* since the lactose dehydrogenase is a membrane-bound system which requires not only the membrane integrity but also cellular viability for cofactor regeneration (Nishizuka and Hayaishi 1962).

Furthermore, the initial pH value plays a key role in fermentation performance, as can be seen in Fig. 2. Initial pH values lower than 5.5 not only delayed the onset of the lactobionic acid production phase, but also inhibited the cellular proliferation phase of *P. taetrolens*. Interestingly, the onset of lactobionic acid production took place without reaching the end of the growth phase or obtaining meaningful cellular densities. In contrast, pH-shift cultivations performed with an initial pH value higher than 7.0 neither provided support for higher cell densities in the stationary growth phase nor triggered lactobionic acid production (Fig. 2a and f). The onset of the lactobionic acid phase was clearly inhibited at initial pH values higher than 7.0, suggesting that *P. taetrolens* cells responded to these deleterious basic conditions with a longer length of the pH-shift (Fig. 2c). It was clear that the control of pH during lactose bioconversion exerted a major influence on the physiology of *P. taetrolens*, revealing that a pH of around 6.5 was optimal both for lactobionic acid production and physiological status. pH values outside the range 6.0–7.0 thus displayed a detrimental influence on fermentation efficiency, since these values involved the prevalence of sub-lethally injured or dead cells during cultivation in contrast to healthy functional cells supported by pH-control strategies at 6.5.

In conclusion, this study has revealed the physiological responses of *P. taetrolens* cells to different pH-control strategies, providing key information for identifying the main deleterious factors affecting bioconversion performance and lactobionic acid production. Both lactobionic acid yield and productivity were strongly affected by the pH-control strategy applied. This concise understanding of the functional and physiological parameters involved has thus enabled the implementation of suitable pH-control modes to prevent potentially deleterious bioprocessing conditions for enhanced lactobionic acid production.

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4.7. Estrategias de alimentación para la mejora en la producción de ácido lactobiónico

En el presente subapartado se ha pretendido mejorar la producción de ácido lactobiónico a partir de suero lácteo con *P. taetrolens*, empleando para ello estrategias sencillas de alimentación en discontinuo. Se ha perseguido alcanzar un rendimiento de producción alto (>90%) empleando técnicas simples de alimentación basadas en el incremento de la disponibilidad de la lactosa en el medio (>100 g/L). Así, se ha evaluado la idoneidad de diferentes estrategias como son la alimentación en contínuo, con multi-pulsos o bien una estrategia de co-alimentacion conformada por un aporte constante de suero lácteo junto con pulsos de una solución concentrada de lactosa. Igualmente, se ha evaluado el status fisiológico de *P. taetrolens* vinculado a tales estrategias de alimentación, sugiriendo las mejores condiciones con el fin de obtener unos niveles de productividad y de producción relevantes a escala industrial. La concentración de ácido lactobiónico obtenida (180 g/L) supera ampliamente los márgenes que Pollard y Woodley (2007) o Yang et al. (2007) consideran como niveles base (80-100 g/L) para empezar a considerar a cualquier sistema biocatalítico de producción industrial como alternativa factible a los sistemas basados en catálisis química. Como se ha mencionado en el subapartado 2.3, es obvio mencionar que los procesos fermentativos en general presentan valores de productividad inferiores a los sistemas enzimáticos. Sin embargo, la complejidad asociada aguas arriba y el altísimo coste de los sistemas enzimáticos disponibles en el mercado impide la total implementación industrial de los mismos.

Asimismo, en este trabajo se realiza una comparación con todos los sistemas microbianos existentes en la bibliografía, enfatizando las características más relevantes como son la eficiencia, la sostenibilidad o el bajo coste del sustrato que conforman el sistema aquí descrito en contraposición con el resto de sistemas fermentativos descritos hasta la fecha.

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Feeding strategies for enhanced lactobionic acid production from whey by *Pseudomonas taetrolens*

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Abstract

High-level production of lactobionic acid from whey by *Pseudomonas taetrolens* under fed-batch fermentation was achieved in this study. Different feeding strategies were evaluated according to the physiological status and fermentation performance of *P. taetrolens*. A lactobionic acid titer of 164 g/L was obtained under co-feeding conditions affording specific and volumetric productivities of 1.4 g/g.h and 2.05 g/L.h, respectively. Flow cytometry assessment revealed that *P. taetrolens* cells exhibited a robust physiological status, which makes them particularly well-suited for employing concentrated nutrient solutions to further prolong the growth and production phases. Such detailed knowledge of the physiological status has been revealed to be a key issue to further support the development of high-yield lactobionic acid production processes under feeding strategies. The present study has demonstrated the feasibility of *P. taetrolens* to achieve high-level bio-production of lactobionic acid from whey through fed-batch cultivation, suggesting its major potential for industrial-scale implementation.

Keywords: lactobionic acid; fed-batch; feeding strategies; flow cytometry; *Pseudomonas taetrolens*.

1. Introduction

Lactobionic acid has recently emerged as a high value-added polyhydroxy bionic acid with a plethora of applications in the food (Gerling, 1998), cosmetics (Green et al., 2009) and pharmaceutical industries (Southard and Belzer, 1995). As an emergent substance, lactobionic acid is also gaining substantial relevance in tissue engineering (Park et al., 2003) and nanomedicine (Peng et al., 2007; Selim et al., 2007) due to its unique biocompatible, biodegradable, chelating, amphiphilic and antioxidant properties. Although lactobionic acid is currently produced at an industrial scale by means of chemical catalysis, its production through microbial fermentation may overcome drawbacks such as the use of polluting catalysts (Kuusisto et al., 2007) or the generation of undesirable side-reaction products associated with the chemical synthesis (Chia et al., 2008).

Unlike current chemically-based processes, bio-production of lactobionic acid through microbial fermentation from an inexpensive feedstock such as cheese whey additionally provides an environmentally-friendly and sustainable alternative (Alonso et al., 2011, 2012a). The use of this high-strength polluting waste as an inexpensive source undoubtedly

opens up the path for cost-effective lactobionic acid manufacturing through microbial fermentation. In this regard, lactobionic acid constitutes a non-growth-associated bio-product resulting from lactose oxidation by *Pseudomonas taetrolens* (Alonso et al., 2011). The deleterious influence of an over-supply of oxygen (Alonso et al., 2012a) as well as the operational pH (Alonso et al., 2012c) have both been identified as key bioprocessing factors affecting bioconversion performance. Likewise, both fermentation performance and lactobionic acid productivity have been shown to be strongly reliant on the overall physiological status displayed by *P. taetrolens* (Alonso et al., 2012b). In fact, physiological heterogeneity within a fermentation system represents a critical parameter that reduces product yield and productivity due to the presence of damaged and dead subpopulations (Hewitt and Nebe-von-Caron, 2004; Müller et al., 2010). The presence of asynchronous cell subpopulations comprising metabolite producing and non-producing cells may alter the product formation pattern and consequently overall bioprocess efficiency (Want et al., 2009). Furthermore, both imbalanced microenvironmental and bioprocessing conditions may involve a stress-induced physiological response with cellular deterioration (Hewitt et al., 1999; Lopes da Silva et al., 2011). Therefore, a precise understanding of the underlying physiological responses to the microenvironmental limitations provides valuable information for further successful implementation of non-deleterious fermentation strategies (Lopes da Silva et al., 2009).

Fed-batch cultivation has become the standard operating mode adopted by industrial biotechnology in order to achieve high-yield metabolite productivity (Ahn et al., 2000; Koller et al., 2008; Yu et al., 2012). However, these cultivation approaches must take physiological information into account, as microorganisms could be submitted to undesired nutrient depletion or metabolic overflow, consequently leading to an irreversible stress-induced physiological response (Amanullah et al., 2002; Hewitt et al., 1999; Want et al., 2009). Such information can be thoroughly addressed by multi-parameter flow cytometry (Díaz et al., 2010; Hewitt and Nebe-von-Caron, 2004), which enables the detection and monitoring of changes in the physiological status of microorganisms. Physiological robustness could thus be deciphered before industrial-scale implementation in order to prevent any potential deleterious influence on the biological performance of microbial cells. Undoubtedly, detailed knowledge about physiological status constitutes a key issue to further support the development of bioprocesses targeting high-yield lactobionic acid production.

The present study evaluates for the first time different feeding strategies, including co-feeding, continuous and multi-pulse feeding conditions, for enhanced lactobionic acid

production from whey by *P. taetrolens*. Fed-batch cultivations were accordingly carried out in order to decipher and establish the most suitable operating approach for improved fermentation performance. Furthermore, the underlying physiological heterogeneity of *P. taetrolens* was also monitored through multi-parameter flow cytometry, thereby addressing the potential deleterious influence of these novel feeding strategies on the physiological status of these microorganisms. Overall, the results may lead to a deeper process understanding for the bio-production of lactobionic acid from whey at an industrially relevant titer.

2. Materials and methods

2.1. Microorganism

Pseudomonas taetrolens LMG 2336, obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium), was maintained frozen (in 40 % [v/v] glycerol at -20ºC). The strain was subsequently subcultured on Nutrient Broth (NB, containing 1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone and 5 g/L NaCl) agar plates, incubated for 48 h at 30ºC, and then preserved at 4ºC.

2.2. Inoculum preparation

A loopful of *P. taetrolens* from a fresh Nutrient Broth agar plate was used to inoculate a 500 mL Erlenmeyer flask containing 100 mL of Nutrient Broth medium. This flask was incubated on an orbital shaker (Infors HT, model Flyer Aerotron, Switzerland) at 250 rpm and 30ºC for 10 h. Actively growing cells from this culture were then employed as inoculum for the production of lactobionic acid in bioreactor cultures containing sweet whey, as subsequently reported.

2.3. Sweet whey preparation

Diluted sweet whey was prepared by diluting sweet cheese whey (provided by ILAS S.A., Asturias, Spain) 1-fold with distilled water (1:1) and adjusted to pH 6.5 (by adding 6 M NaOH) prior to sterilization using a tangential microfiltration device equipped with a 0.22 μm pore size PVDF membrane-cassette (Millipore, Massachusetts, USA). Concentrated cheese whey was prepared as above by microfiltration of undiluted sweet cheese whey. A highly concentrated whey solution was prepared as above by microfiltration of undiluted sweet cheese whey supplemented with lactose (82 g/L), peptone (2.5 g/L) and yeast extract (2.5 g/L).

2.4. Bioreactor cultivations

Bioreactor cultivations were performed in a 2-L bioreactor (BioFlo 110, New Brunswick Scientific, NJ, USA) with an initial working volume of 1 L at 30ºC and an agitation rate of 350

rpm. Besides, the bioreactor was aerated (1 Lpm) via a ring sparger located at the bottom of the vessel. Excessive foam formation was prevented by automatic addition of diluted (1:10) Y-30 emulsion (Sigma-Aldrich, Steinheim, Germany). The bioreactor was equipped with a pH meter (Mettler Toledo, Switzerland) and a polarographic dissolved oxygen electrode (InPro 6830, Mettler Toledo, Switzerland) for continuous on-line monitoring of pH and dissolved oxygen tension (DOT) values, respectively. A two-stage pH-shifted bioconversion strategy was adopted as previously reported (Alonso et al. 2011). The strategy consisted in controlling pH above 6.5 (pH was left uncontrolled above this value during the growth phase and then maintained at 6.5) by means of computer-controlled peristaltic pumps via automatic addition of 4 M NaOH. These prior conditions were applied to all cultivations unless otherwise specified. Cultivations were carried out in duplicate as independent experiments.

Batch cultivation for lactobionic production from undiluted sweet whey was conducted with an inoculation level of 30% (v/v). The seed culture was obtained by harvesting biomass (after centrifugation at 11,000x*g* for 10 min) from a NB shake-flask culture and re-suspending cells in a 500 mL Erlenmeyer flask containing 100 mL of diluted sweet whey. These cultures were subsequently grown at 250 rpm in an orbital shaker at 30ºC for 12 h and then employed as seed culture in the bioreactor at 30% (v/v) containing undiluted sweet whey.

2.5. Influence of feeding rates on fermentation performance

The influence of different feeding rates (4.2, 8.4, 16.8, 33.6 and 67.2 mL/h) on bioprocess parameters in combination with a pH-shift control strategy was evaluated. A total volume of 0.3 L of highly concentrated whey solution were thus continuously added at different flow rates into bioreactor containing diluted sweet whey. Fed-batch cultivations were conducted with an inoculation level of 10% (v/v), in which the DOT was maintained at 10% via an agitation cascade (from 350 to 500 rpm) during the growth phase.

2.6. Influence of multi-pulse feeding conditions

In order to gain insight into how system and bioprocess parameters are affected by the availability of different lactose levels, cultivation was submitted to multi-pulse feedings of highly concentrated lactose solution (275 g/L). Pulses of 0.1 L were performed at either 10, 24 or 34 h. Fed-batch cultivation was conducted at an inoculation level of 30% (v/v), maintaining the DOT level above 10% via an agitation cascade (from 350 to 500 rpm).

2.7. Fed-batch cultivations under a co-feeding strategy

Fed-batch cultivations were carried out under a co-feeding strategy which consisted in combining multi-pulse feeding with lactose (every pulse of 0.1 L contained 275 g/L of

lactose) with continuous feeding of highly concentrated whey (a total volume of 0.3 L containing 160 g/L of lactose, 2.5 g/L of yeast extract and 2.5 g/L of peptone) at rates of 4.2 and 33.6 mL/h. The pulse feeding of the high-lactose solution (275 g/L) was carried out at regular intervals (10, 24 and 34 h). Fed-batch cultivations were conducted at an inoculation level of 30% (v/v), maintaining the DOT level above 10% via an agitation cascade (from 350 to 500 rpm).

2.8. Staining procedures and multi-parameter flow-cytometry

Samples from cultures were harvested by centrifugation at 16,000x*g* for 5 min. Before staining, cells were washed twice in phosphate-buffered saline (PBS, pH 7.4, sterile and filtered at 0.22 μm) and were then held in the "hot spot" of a sonication bath for 2 s to prevent bacterial aggregation before flow cytometric analysis (Hewitt and Nebe-von-Caron, 2004). Propidium iodide (PI, Invitrogen), bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol, $DiBAC_4(3)$, Invitrogen) and carboxyfluorescein diacetate (cFDA, Invitrogen) were used as fluorescent dyes in a double dual-staining procedure ($DiBAC_4(3)$/PI and cFDA/PI) to evaluate cell physiological status (metabolic activity, membrane integrity and membrane polarization were evaluated through cFDA, PI and $DiBAC_4(3)$ staining, respectively). Staining solutions were prepared and procedures were conducted as previously reported by Alonso et al. (2012b).

Flow cytometry measurements were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with a 488 and 633 nm excitation light source from an argon ion laser. Green fluorescence from samples (corresponding to $DiBAC_4(3)$ and cFDA-stained cells) was collected on the FL1 channel (530 nm), whereas PI fluorescence was registered on the FL3 channel (610 nm). Each analysis was performed in duplicate at a low flow rate setting (4000 events/s). Data acquisition was carried out using Cytomics RXP software (Beckman Coulter). Gates and quadrants were established according to staining controls. For $DiBAC_4(3)$/PI and cFDA/PI dual-parameter flow cytometric analysis, data respectively collected from 150,000 and 100,000 events were analyzed using Summit v4.3 software (DakoCytomation, Colorado, USA).

2.9. Analytical methods

Bacterial growth was measured spectrophotometrically as optical density at 600 nm (Shimazdu, UV 1203 model) after centrifugation of culture samples at 16,000x*g* for 5 min. Optical density data was converted to cell dry weight (expressed in grams per litre) using the corresponding previously obtained calibration curve. The lactobionic acid and lactose content of cell-free culture samples were measured by high performance liquid chromatography as reported previously by Pedruzzi et al. (2007). The liquid

chromatography system used for analysis (Agilent 1200, Agilent Technologies Inc., California, USA) was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., California, USA) coupled to a refractive index detector. Sulphuric acid (0.450 mM, pH 3.1) was employed as the mobile phase at a flow rate of 0.3 mL/min with the column temperature set at 75ºC. Data acquisition and analysis were performed with ChemStation software (Agilent).

2.10. Determination of the oxygen uptake rate

The oxygen uptake rate (OUR) from bioreactor cultivations was determined via the dynamic method. The OUR was accordingly obtained from the slope of the plot of dissolved oxygen concentration versus time following a momentary interruption of air supply to the bioreactor (Alonso et al. 2012a). The specific consumption rate of oxygen (qO_2) was calculated by dividing the OUR value by the biomass concentration at each sampling point.

3. Results and discussion

3.1. pH-shift batch cultivation on concentrated whey

As can be seen in Fig. 1A, a lactobionic acid titer of 78.4 g/L with a volumetric productivity of 1.63 g/L.h was obtained under batch cultivation using concentrated whey. These levels respectively represent 1.9- and 2.3-fold enhancement compared to the titer and productivity values obtained under batch cultivation with diluted whey medium (Alonso et al., 2011), subsequently achieving complete lactose bioconversion (100%) after 48 h (Fig. 1A). The onset of the stationary growth phase featured the usual increase in DOT values concomitantly to an abrupt drop in pH of the culture broth (Alonso et al., 2011). Interestingly, *P. taetrolens* cells displayed a specific productivity value of 1.02 g/gDCW.h, which is significantly higher than that obtained in diluted whey media under high-cell density conditions, namely 0.92 g/gDCW.h (Alonso et al., 2012a). Upon the onset of the lactobionic acid production phase, a return of dissolved oxygen to saturated values was observed along with a decreased specific oxygen uptake rate at a late stage (~10 mg/gDCW.h).

Figs. 1B and 1C show the changes in the physiological status of *P. taetrolens* in terms of metabolic activity, membrane integrity and polarization during the pH-shift batch cultivation on concentrated whey. As previously reported, the physiological heterogeneity of *P. taetrolens* plays a crucial role in lactobionic acid bio-production (Alonso et al., 2012b). The presence of both an intact and polarized cytoplasmic membrane and functional nucleic acids undoubtedly represent the key parameters of cellular viability (Hewitt and Nebe-von-Caron, 2004). As can be seen in Figs. 1B and C, *P. taetrolens* cells were not induced towards a

stress physiological response during the lactobionic acid production phase in batch bioreactor cultivation, remaining predominantly metabolically active (~65%). This result contrasts with shake-flask cultivation, in which prolonged exposure to stressful bioprocessing conditions led to a loss in cellular functionality (Alonso et al., 2012b).

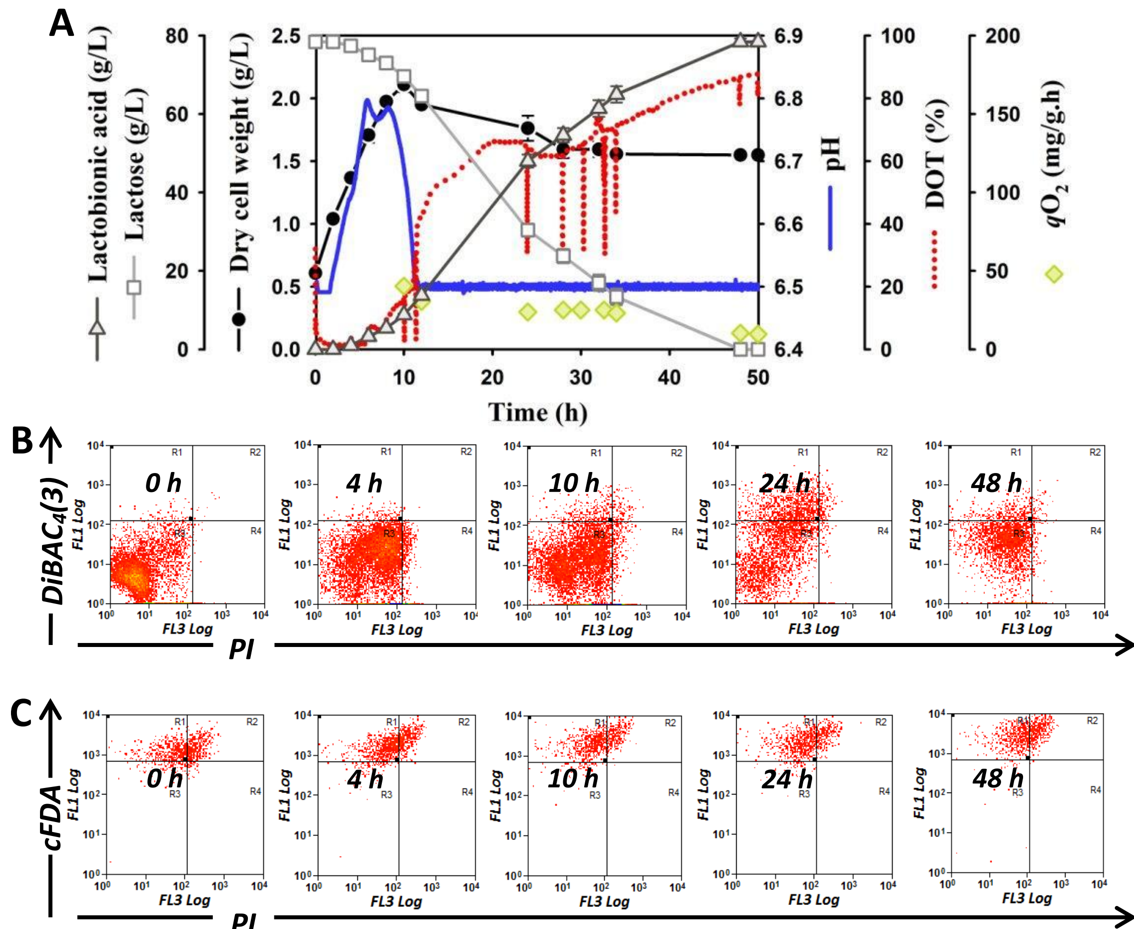

Figure 1. Bioprocess parameters (A), dot plots representing DiBAC$_4$(3) fluorescence versus PI fluorescence (B) and cFDA fluorescence versus PI fluorescence signals (C) obtained during pH-shift bioreactor cultivation of *P. taetrolens* on concentrated whey. The upper left quadrant of the dot plots in B shows depolarized cells (DiBAC$_4$(3)-positive; DiBAC$_4$(3) enters cells only when the membrane potential is lost), while cells with esterase activity (cFDA-positive cells; cFDA is cleaved by the esterase activity inside the living cells, thus releasing a polar fluorescent portion which is unable to pass through the intact membrane) are shown in the upper left quadrant of C. Damaged cells (corresponding to cFDA(+)/PI(+) cells or DiBAC$_4$(3)(+)/PI(-) cells) are shown in the upper left quadrant of B and in the upper right quadrant of C for the DiBAC$_4$(3)/PI and cFDA/PI staining, respectively. Likewise, dead cells (PI-positive cells; PI can only cross the plasmatic membrane if it is permeabilized corresponding to those cells whose membranes are compromised) are shown in the upper right quadrant and in the lower right quadrant for the DiBAC$_4$(3)/PI (B) and cFDA/PI staining (C), respectively.

Unlike healthy functional cells, damaged and dead cells suffer structural changes associated with the collapse of cellular functions, including the proton motive force (Díaz et al., 2010; Müller et al., 2010). In fact, stress-induced physiological responses due to inappropriate bioprocessing conditions may directly result in reduced fermentation efficiency and biological performance (Lopes da Silva et al., 2009). While the growth phase feature a high percentage of metabolic activity (>90%), the transient phase between the growth and production periods featured a loss in membrane polarization, as shown in Fig. 1B, remaining mainly depolarized (~70%) during the stationary phase. Moreover, flow cytometric assessment revealed the presence of two distinct cell subpopulations, as the dot plots in Fig. B show, although this fact did not lead to asynchronous lactobionic acid production.

3.2. Impact of the feeding rate on lactobionic acid production

Fig. 2 shows the influence of different constant feeding rates on bioprocess parameters aimed at improving the lactobionic acid titer and yield. Feeding was completed during the exponential growth phase at the highest rate (67.2 mL/h for 4.5 h) in contrast with the continuous feeding strategy adopted under the lowest rate (4.2 mL/h for 72 h). Table 1 summarizes the overall cultivation parameters obtained with the five different feeding rates employed. As can be seen, higher feeding rates stimulated the growth of *P. taetrolens* cells, resulting in improved proliferation patterns (Fig. 2A). The highest maximum biomass concentration (2.15 g/L) and specific growth rate (0.16) were obtained under the influence of the highest rates (33.6-67.2 mL/h) (Table 1), suggesting that increasing feeding rates contributed not only to higher biomass, but also to higher specific growth rates. Dissolved oxygen profiles showed similar trends in the fed-batch cultivations (Fig. 2B), in which DOT levels were maintained at 10% during the growth phase by means of an agitation cascade (from 350 to 500 rpm), as oxygen-limited conditions during this phase improve the fermentation performance of *P. taetrolens* cells (Alonso et al., 2012a). pH-shift values were correlated with feeding rates (Fig. 3), with increasing rates leading to a progressive rise in pH-shift values (7.52, 7.64, 7.74, 7.90 and 7.92 for 4.2, 8.4, 16.8, 33.6 and 67.2 mL/h, respectively) (Fig. 2C). Increased feeding rates reduced oxygen consumption by *P. taetrolens* cells, as indicated by a decrease in OUR values during the lactobionic acid production phase (Fig. 2D). Whereas lower OUR values (~7 mg/L.h) were found at higher rates (16.8-67.2 mL/h), a feeding rate of 4.2 mL/h supported values of even 25 mg/L.h at a late cultivation stage. These results suggest that the higher nutrient availability found under the continuous

feeding rate (4.2 mL/h) led to elevated metabolic activity of *P. taetrolens* cells during the lactobionic acid production phase.

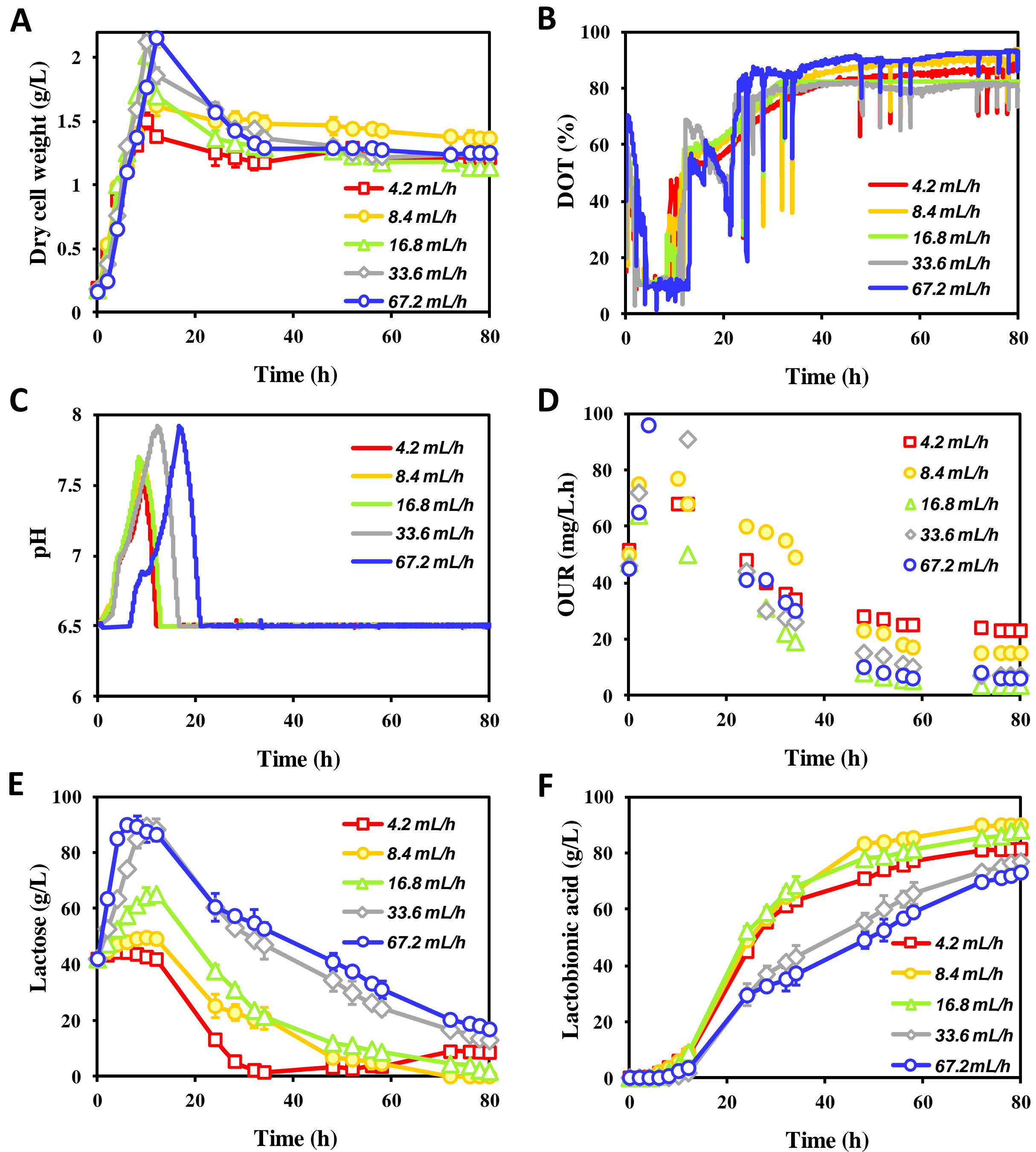

Figure 2. Effect of different constant feeding rates (4.2, 8.4, 16.8, 33.6 and 67.2 mL/h) on *P. taetrolens* fed-batch cultivations. Time-course profiles of cell growth (A), DOT (B), pH (C), OUR (D), lactose (E) and lactobionic acid (F) are shown.

Unlike cellular proliferation, lactobionic acid production was negatively affected by higher feeding rates (Fig. 3). The use of feeding rates higher than 8.4 mL/h was therefore detrimental to lactose oxidation, leaving residual lactose contents of 2, 13 and 17 g/L after 80 h for rates of 16.8, 33.6 and 67.2 mL/h, respectively (Fig. 2E). Despite the higher lactose availability in the fermentation broth (around 90 g/L) at rates of 33.6 and 67.2 mL/h, lactose oxidation into lactobionic acid was negatively affected, suggesting that 8.4 mL/h was the optimal rate, since it met the specific requirements for lactose oxidation by *P. taetrolens* cells, as can be seen in Figs. 2E and F. Note that a feeding rate of 4.2 mL/h led to nearly complete exhaustion of the lactose content at 34 h which was detrimental for further oxidation of the lactose added via this continuous feeding strategy (Fig. 2E), resulting in a bioconversion yield of 90% compared to the complete bioconversion (100%) achieved at 8.4 mL/h (Table 1). This latter fact might suggest that induction of lactose oxidation in *P. taetrolens* cells needs a lactose level higher than 5 g/L in the fermentation broth. The overall fermentation performance in terms of lactobionic acid productivity was clearly reduced under increased feeding rates, resulting in a volumetric productivity of 0.97 g/L.h obtained at a rate of 67.2 mL/h versus a productivity of 1.25 g/L.h at a rate of 8.4 mL/h (Fig. 3).

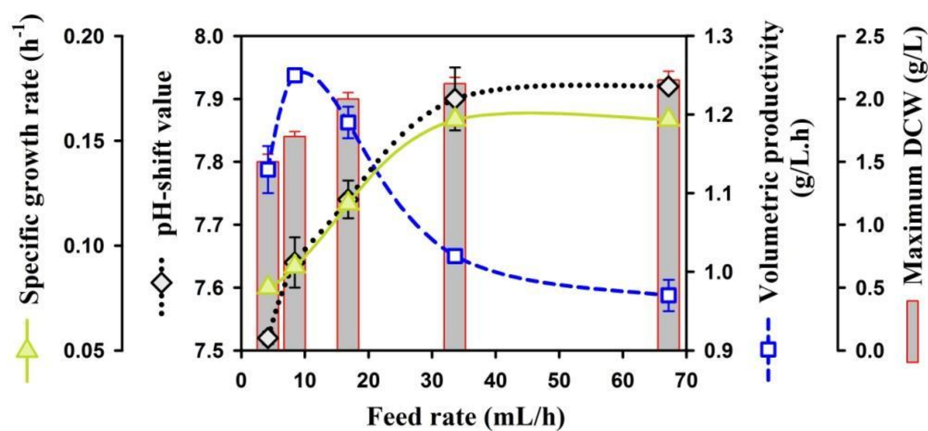

Figure 3. Relationship between feeding rates and specific growth rate, maximum biomass (expressed as Dry Cell Weight, DCW), volumetric productivity and pH-shift values obtained during fed-batch pH-shift cultivations at 6.5.

Table 1. Summary of values obtained in pH-shift fed-batch cultivations under the influence of different feeding rates.

Feeding rate (mL/h)	*Maximum dry cell weight (g/L)*	*Duration of the growth phase (h)*	*Onset of the production phase (h)*	*pH-shift value*	*μ (h^{-1})*	*Volumetric productivity (g/L.h)*	*Yield (%)* [a]
4.2	1.50 ± 0. 06	10	6	7.52 ± 0. 01	0.08	1.13 ± 0. 03	90 ± 2
8.4	1.70 ± 0. 04	10	6	7.64 ± 0. 04	0.09	1.25 ± 0. 00	100 ± 0
16.8	2.01 ± 0. 05	10	8	7.74 ± 0. 03	0.12	1.19 ± 0. 02	95 ± 2
33.6	2.12 ± 0. 05	10	10	7.90 ± 0. 05	0.16	1.02 ± 0. 00	82 ± 0
67.2	2.15 ± 0. 07	12	12	7.92 ± 0. 00	0.16	0.97 ± 0. 02	77 ± 2

[a] Yield was defined as the percentage of lactose converted into lactobionic acid after 72 h.

μ = Specific growth rate

3.3. Influence of multi-pulse feeding conditions on lactobionic acid production

As Fig. 4 shows, a multi-pulse feeding mode was conducted to further improve lactobionic acid titer as well as to simultaneously examine the influence of this high-yield production on the physiological status of *P. taetrolens*. A highly concentrated lactose solution was accordingly fed into the bioreactor at different time points (10, 24 and 34 h) before the initial content was depleted, thus maintaining a lactose level of around 80 g/L in the fermentation broth (Fig 4A). This lactose pulse feeding strategy involved a momentary depletion in dissolved oxygen values, as can be seen in the DOT profile, which quickly decreased to lower levels after the pulse and then increased progressively to saturated values. As can be seen, *P. taetrolens* cells retained their bioconversion ability throughout the cultivation, leaving only 9 g/L of residual lactose after 80 h, thus enabling both high-yield production (94%) as well as high-level productivity (1.8 g/L.h) from whey (Table 2). *P. taetrolens* cells also displayed a relatively high specific oxygen consumption rate (~30 mg/gDCW.h) at the mid-stationary phase, although the late cultivation stage featured lower consumption rates (~16 mg/gDCW.h) (Fig. 4A).

As regards the physiological status of *P. taetrolens*, the feed pulse did not exert a major deleterious influence on the membrane integrity of the cells, as the dot plots in Figs 4B and C show. Cells remained predominantly healthy and metabolically active (70%) from the end of exponential growth phase onwards (Fig. 4C), despite the high lactose content of the pulse feed (275 g/L), suggesting that cells did not suffer any resulting stress-induced physiological response. The onset of the production phase featured progressive depolarization (85% from 34 onwards), although the cells retained their membrane integrity throughout the

cultivation (Fig. 4B). This robust physiological status exhibited by *P. taetrolens* cells makes them particularly well-suited for employing concentrated nutrient solutions to further prolong the growth and/or production phases. The loss in the cellular functionalities of *P. taetrolens* cells could lead to a decline in their lactobionic acid producing ability due to the presence of undesired damaged and dead subpopulations (Alonso et al., 2012b).

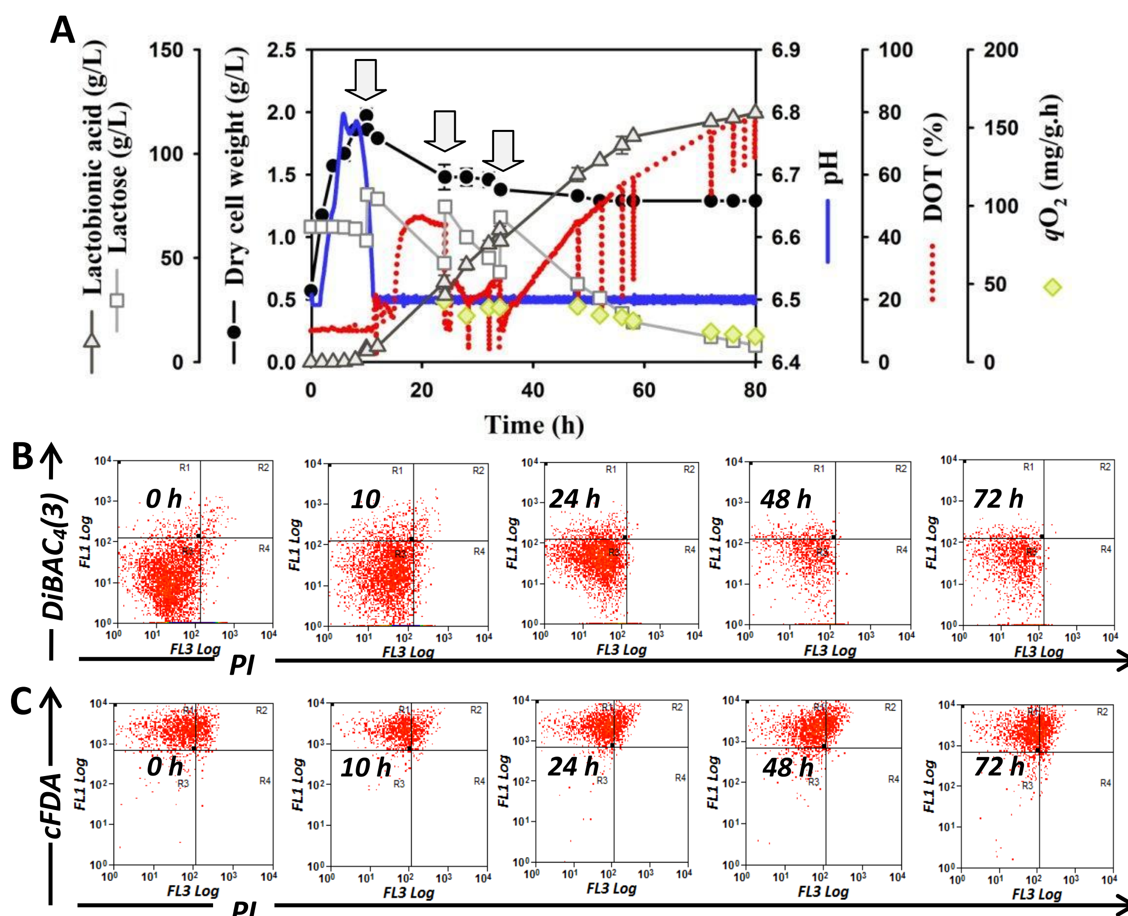

Figure 4. Time-course profile of bioprocess parameters (A) and dot plots representing DiBAC$_4$(3)/PI (B) and cFDA/PI fluorescence signals (C) during multi-pulse fed-batch cultivation. Arrows indicate the time point of pulse feeding with 0.1 L of 275 g/L lactose.

3.4. Co-feeding strategy for enhanced lactobionic acid production

In order to achieve high-level production of lactobionic acid, cultivations were submitted to a novel co-feeding strategy combining continuous feeding of concentrated whey (performed at two different rates, 4.2 and 33.6 mL/h) with multi-pulse feedings of highly concentrated lactose solutions. Specifically, highly concentrated whey solutions (Ahn et al., 2000) in combination with nutrient supplementation (Koller et al., 2008; Nath et al., 2008)

are usually employed to achieve enhanced metabolite production from whey in fed-batch cultivations. Supplementary nitrogen sources such as yeast extract and peptone do in fact promote *P. taetrolens* growth, since they provide vitamins, trace elements and amino acids as carbon source which support cellular growth (Matsui et al., 2009). As can be seen in Fig. 5A, the co-feeding strategy at a rate of 4.2 mL/h displayed a higher specific growth rate (0.21 h^{-1}) compared to batch cultivation (0.12 h^{-1}), although the maximum cell biomass resulted in similar values (around 2.1 g/L). Furthermore, similar maximum cell biomass values were comparatively achieved between the two feeding rates tested, 4.2 and 33.6 mL/h, although the former rate supported a higher biomass during the lactobionic acid production phase due to the continuous feeding strategy (Fig. 6A).

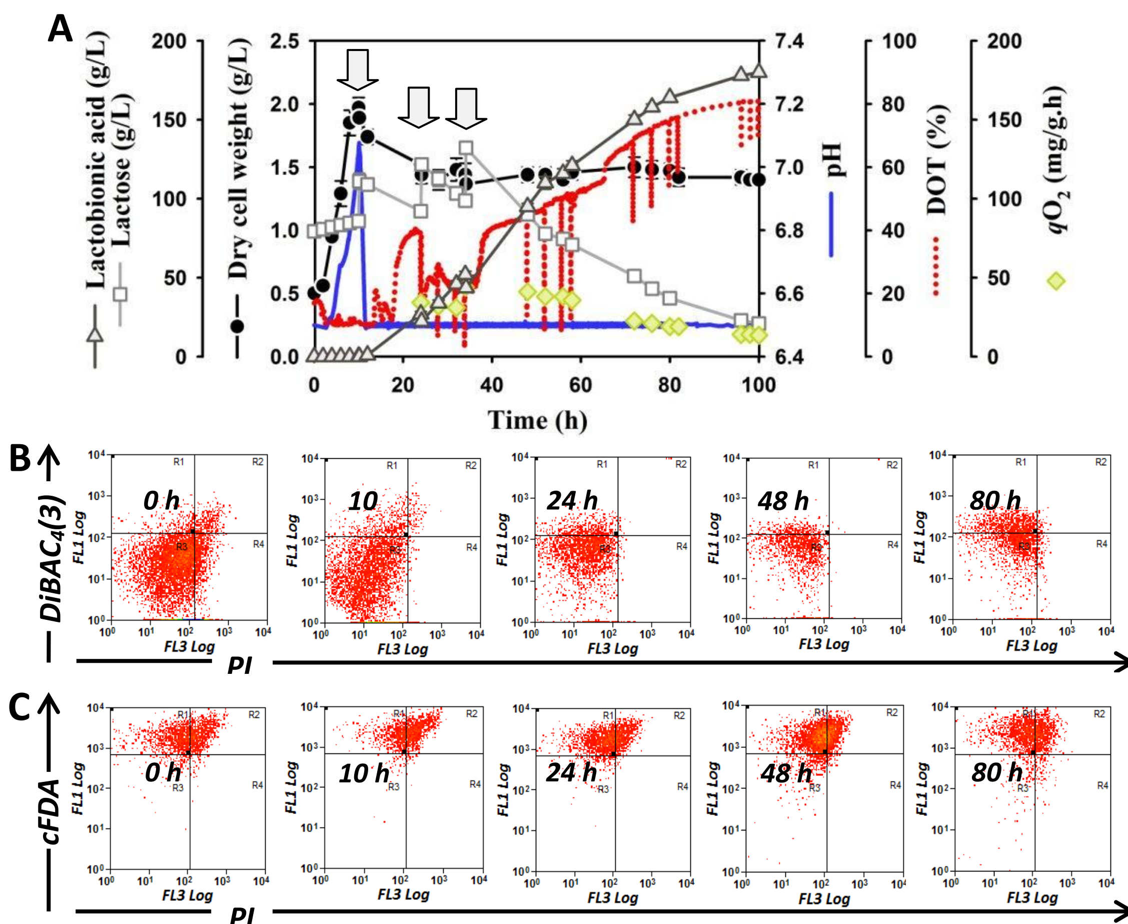

Figure 5. Bioprocess parameters (A) and physiological status of *P. taetrolens* from DiBAC$_4$(3)/PI (B) and cFDA/PI staining (C) during fed-batch cultivation performed under a co-feeding strategy at a rate of 4.2 mL/h. Arrows indicate the time point of pulse feeding with highly concentrated lactose solution (0.1 L of 275 g/L).

The operational life span of the fed-batch fermentations also increased in comparison with batch cultivation using concentrated sweet whey (100 versus 48 h) (Fig. 1). However, the growth phases were not prolonged under these co-feeding strategies, as Figs. 5A and 6A show. The DOT profile in fed-batch cultivations performed at 33.6 mL/h returned to saturated values after completing the feed (4.5 h) in contrast with the cultivation carried out under a continuous feeding rate, in which the oxygen-period was prolonged for 17 h (Figs. 5A and 6A). Besides, an abrupt decline in the DOT profile was found at 4.2 mL/h in response to the lactose feeding pulses, whereas the absence of DOT responses at 33.6 mL/h indicated that the cultivation was operating under lower nutrient availability. As regards the oxygen consumption, plateaus in the specific oxygen uptake rate were reached simultaneously in both cases with the onset of lactobionic acid formation.

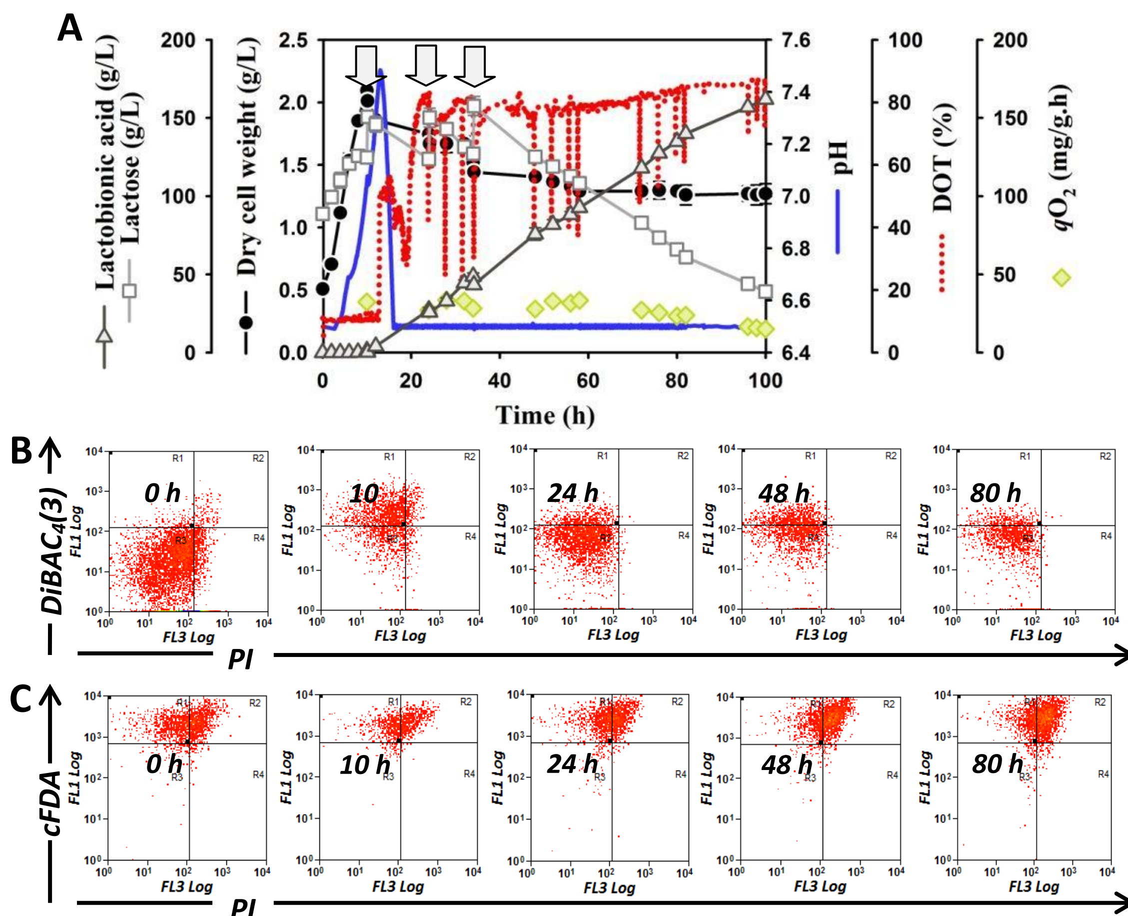

Figure 6. Time-course profile of bioprocess parameters (A) and dot plots representing DiBAC$_4$(3)/PI (B) and cFDA/PI fluorescence signals (C) during fed-batch cultivation performed under a co-feeding strategy at a rate of 33.6 mL/h. Arrows indicate the time point of pulse feeding with highly concentrated lactose solution (0.1 L of 275 g/L).

Fermentation performance was clearly enhanced under the co-feeding strategy at 4.2 mL/h, in which a lactobionic acid titer of 180 g/L and a yield of 90% were obtained after 100 h, compared to a titer of 162 g/L and a yield of 80% obtained under co-feeding at 33.6 mL/h. The adopted feeding strategy led to an uncoupling of lactobionic acid formation from cell growth, as can be seen in the cellular growth profiles of *P. taetrolens* (Figs. 5A and 6A). Moreover, the co-feeding strategy at 4.2 mL/h maintained the lactose level in the fermentation broth above 100 g/L (Fig. 5A), in contrast with the 150 g/L maintained under a co-feeding strategy at 33.6 mL/h (Fig. 6B), thus suggesting that higher lactose availability levels in the fermentation broth did not stimulate lactobionic acid production.

Comparatively, the co-feeding strategy at 4.2 mL/h presented an enhancement in terms of productivity (1.13- and 1.8-fold higher than the continuous and the multi-pulse feeding strategy, respectively) and lactobionic acid titer (180 versus 81 or 143 g/L under continuous and multi-pulse feeding, respectively). Whereas a yield of 94% was obtained under a fed-batch process with multi-pulse lactose feeding, a yield of 82% was only obtained under co-feeding conditions (Table 2). These results demonstrate that these fed-batch cultivation approaches enhanced microbial culture performance as well as increasing the lactobionic acid titer.

Table 2. Comparison of bioprocess parameters, lactobionic acid productivity and titer values obtained under different feeding strategies.

Feeding strategy	*Maximum dry cell weight (g/L)*	*Duration of the growth phase (h)*	*Onset of the production phase (h)*	*pH-shift value*	*μ (h^{-1})*	*Volumetric productivity (g/L.h)*	*Culture time (h)*	*Yield (%)* [a]
Continuous [b]	1.50 ± 0. 06	10	8	7.52 ± 0. 01	0.08	1.13 ± 0. 03	72	90 ± 2
Multi-pulse	1.97 ± 0. 05	10	12	6.82 ± 0. 05	0.04	1.80 ± 0. 02	80	94 ± 1
Co-feeding [c]	2.05 ± 0. 08	10	12	7.08 ± 0. 05	0.21	2.05 ± 0. 02	72	82 ± 2

[a] Yield was defined as the percentage of lactose converted into lactobionic acid after each culture time.

[b] Feeding rate of 4.2 mL/h on diluted whey

[c] Feeding rate of 4.2 mL/h on concentrated whey

μ = Specific growth rate

Regarding the physiological status of *P. taetrolens*, cells remained polarized during the growth phase, whereas a damaged subpopulation appeared at the late cultivation stage (Fig. 5C and 6C). Despite the high lactobionic acid productivity achieved under co-feeding conditions, cells remained depolarized throughout fed-batch cultivation (85%). The feed pulse neither induced major changes in the physiological status displayed by cells nor influenced fermentation performance or lactobionic acid productivity (Fig. 5B and 6B).

Comparatively, the main subpopulation (70%) was damaged at late cultivation stages under the co-feeding mode at 33.6 mL/h (Fig. 6C) versus a higher metabolic status (50%) under continuous co-feeding at 4.2 mL/h even at late stages due to higher nutrient availability (Fig. 5C). The co-feeding strategy thus led to a robust physiological status, suggesting that these bioprocessing conditions did not exert any potential deleterious influence on the cellular functionality of *P. taetrolens*. Undoubtedly, the co-feeding mode at the lowest rate (4.2 mL/h) was able to cope with the cellular responses and achieved a balance between cellular maintenance and lactobionic acid production. The nutrient depletion suffered in the co-feeding mode at the highest rate (33.6 mL/h) could induce considerably higher cell stress and therefore less cellular robustness in terms of physiological status.

Table 3 shows the comparison of different microbial fermentation approaches carried out for the bio-production of lactobionic acid, including whole-cell and permeabilized-cell bioconversion systems. In terms of productivity, the values obtained in the present study (2.05 g/L.h) were higher than those obtained by Miyamoto et al. (2000) (1.87 g/L.h). In this latter study, *Pseudomonas* sp. LS13-1 mutants were able to produce 290 g/L of lactobionic acid from complex nutrient media with a yield of 90% in a fed-batch cultivation supplemented with 15 g/L of peptone as nitrogen source (Miyamoto et al., 2000). Mutants of *Burkholderia cepacia* were additionally found to produce up to 150-400 g/L of lactobionic acid with high volumetric productivities of 1.67-5.55 g/L.h on complex culture media over periods of 27-240 h under resting-state conditions at a shake-flask scale (Murakami et al., 2002, 2006). Despite incomplete lactose bioconversion (85%), a high-level lactobionic acid titer of 178 g/L and a productivity of 3.56 g/L.h were also achieved by *B. cepacia* on a complex culture medium (Meiberg et al., 1990). Nevertheless, the pathogenicity of *B. cepacia* discourages any industrial-scale implementation of this microbial platform as a bionic acid-producing microorganism.

Permeabilized resting free-cell systems of *Zymomonas mobilis* have also been used to obtain high-level production of lactobionic acid from lactose and fructose solutions (Malvessi et al., 2012; Pedruzzi et al., 2011). Comparatively, the volumetric productivities obtained under resting-state conditions in these systems were 2.8- and 3.7-fold higher, respectively, than that obtained in the present study under co-feeding conditions (2.05 g/L.h). Interestingly, the lactobionic acid titers obtained in the present study were 1.4-fold higher than that obtained by Pedruzzi et al. (2011), who reported around 125.4 g/L of lactobionic acid from 252 g/L of chemically pure lactose solution (Table 3). The specific lactobionic acid productivity obtained in the present study (1.4 g/gDCW.h) is the highest reported to date for submerged microbial bioprocesses targeting the production of this

polyhydroxy bionic acid. In this regard, bioconversion systems employing permeabilized resting cells of *Z. mobilis* featured specific productivities of 0.3 (Malvessi et al., 2012) and 0.8 g/gDCW.h (Pedruzzi et al., 2011). These results were substantially lower than that obtained in the present study (1.4 g/gDCW.h), which thus represents an efficient rate of bioconversion ability by *P. taetrolens* cells (Table 3).

As previously highlighted, a distinctive feature of the present fermentation approach is the use of cheese whey as an inexpensive feedstock for lactobionic acid production. In this study, a novel co-feeding strategy was developed to achieve high-level lactobionic acid production from concentrated cheese whey without costly nutrient supplements. Fermentation efficiency in terms of volumetric productivity was clearly enhanced, being 2.9-fold higher than that obtained in batch cultivation at bioreactor (Alonso et al., 2011) or shake-flask scale (Alonso et al., 2012b) (2.05 versus 0.70 g/L.h for fed-batch and batch cultivations, respectively) (Table 3). Furthermore, the lactobionic acid titer (164 g/L) was 3.4-fold higher than that obtained in batch cultivation (42 g/L) under high-cell density conditions on diluted whey (Alonso et al., 2012a). The present study also examined the cellular functionality of *P. taetrolens* cells during fed-batch cultivations. In this regard, fed-batch approaches are applied to obtain high production yields of target metabolites (Tang et al., 2009; Yu et al., 2012). However, these bioprocessing conditions may induce undesired microbial physiological responses which could have a marked impact on overall system performance. Thus, the monitoring of cellular physiology may specifically provide valuable information to improve biological performance, thereby achieving higher biological efficiencies as well as enhanced lactobionic acid production yields by *P. taetrolens* (Alonso et al., 2012b, 2012c). Such detailed knowledge of physiological heterogeneity may also provide valuables guidelines for the design of improved formulations and cultivation strategies (Amanullah et al., 2002; Lopes da Silva et al., 2011; Want et al., 2009). Results from the present study have revealed that a co-feeding strategy with highly concentrated whey solution did not exert a major deleterious influence on the physiological status of *P. taetrolens* cells. Lactobionic acid-producing cells retained their membrane integrity and functionality throughout fed-batch cultivations, although multi-parameter flow cytometry assessment enabled the detection of an increase in the depolarization level which was associated with nutrient exhaustion following the feeding pulse.

Table 3. Comparison of research studies on the microbial production of lactobionic acid.

Microorganism	***Culture medium***	***Cultivation strategy and operational mode***	***Culture time (h)***	***Lactobionic acid titer (g/L)***	***Specific productivity (g/g.h)***	***Productivity (g/L.h)***	***Yield (%)*** [a]	***Reference***
Pseudomonas sp. LS13-1 [b]	Lactose, salts and peptone	Fed-batch in bioreactor	155	290	-	1.87	90	Miyamoto et al. (2000)
Burkholderia cepacia	Lactose, salts, peptone, yeast extract and glucose	Fed-batch in bioreactor	50	178	-	3.56	85	Meiberg et al. (1990)
Burkholderia cepacia No. 24 [b]	Lactose, salts, peptone and yeast extract	Fed-batch in shake-flask	240	400	-	1.67	100	Murakami et al. (2003)
Burkholderia cepacia No. 24 [b]	Lactose, salts, corn steep liquor and yeast extract	Batch in shake-flask	27	150	-	5.55	~100	Murakami et al. (2006)
Zymomonas mobilis [c]	Lactose	Batch in bioreactor	22	125	0.80	5.80	100	Pedruzzi et al. (2011)
Zymomonas mobilis [c]	Lactose, fructose	Batch in bioreactor	24	182	0.30	7.60	78	Malvessi et al. (2012)
Pseudomonas taetrolens	Whey	Batch in bioreactor	58	42	0.56	0.70	100	Alonso et al. (2011)
Pseudomonas taetrolens	Whey	Batch in bioreactor	30	42	0.94	1.27	100	Alonso et al. (2012a)
Pseudomonas taetrolens	Whey	Batch in shake-flask	60	42	0.42	0.70	100	Alonso et al. (2012b)
Pseudomonas taetrolens	Concentrated whey	Batch in bioreactor	48	78	1.02	1.63	100	This study
Pseudomonas taetrolens	Concentrated whey	Fed-batch in bioreactor	80	164	1.40	2.05	82	This study

-: not available

[a] Yield was defined as the percentage of lactose converted into lactobionic acid after each cultivation time.

[b] Mutant strain.

[c] Permeabilized resting free-cells.

4. Conclusions

The present study has demonstrated the feasibility of whey as an inexpensive source for lactobionic acid bio-production by *P. taetrolens* at an industrially relevant titer. A high-level titer of 180 g/L was obtained with a yield of 90% via fed-batch cultivation carried out under co-feeding conditions. Moreover, the physiological responses of *P. taetrolens* cells were monitored through flow cytometry in order to assess the impact of different feeding strategies on bioprocess efficiency. High-yield bio-production of lactobionic acid was directly linked to the fully functional status of *P. taetrolens*, thus providing relevant information for successful industrial implementation.

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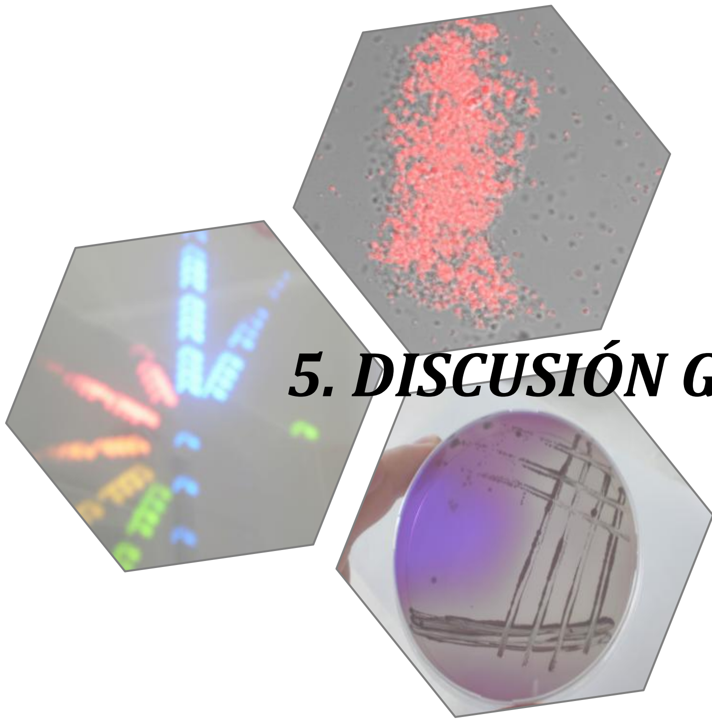

5. DISCUSIÓN GENERAL

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5.1. Producción biotecnológica de ácido láctico

Diferentes materias primas procedentes de subproductos de origen alimentario han sido materia de estudio con el fin de ser utilizadas como sustrato fermentativo, desde cereales caso de la cebada o el trigo (Hofvendahl y Hahn-Hägerdal, 2000; Oh et al., 2005), residuos agrícolas como el salvado de arroz (Altaf et al., 2004), o incluso hasta zumo de dátiles (Nancib et al., 2001). Así, las últimas tendencias en materia de investigación en este campo van encaminadas en la sacarificación (degradación de la biomasa compleja) bien sea a través de hidrólisis ácida o enzimática y posterior fermentación (John et al., 2009). Ambas etapas pueden ser llevadas a cabo a través de una hidrólisis y fermentación o como sacarificación simultánea y posterior fermentación. De hecho, este proceso de fermentación está íntimamente ligado a la producción de ácido láctico a partir de materias con alto contenido en almidón (caso de la pulpa de mandioca, almidón de patata, salvado de harina o almidón de maíz) y de materiales lignocelulósicos (tales como la celulosa o residuos madereros) utilizando microorganismos amilolíticos. No obstante, el empleo de esta metodología encarece los procesos de obtención de ácido láctico. Recientemente, los esfuerzos están centrados en la búsqueda de microorganismos capaces de realizar simultáneamente una primera sacarificación y posterior fermentación de los carbohidratos resultantes. De hecho, los esfuerzos relacionados con la producción biotecnológica de ácido láctico van encaminados actualmente en dos direcciones: mejora en las capacidades funcionales de los microorganismos a través del aislamiento de muevas cepas amilolíticas o bien en la utilización de nuevas fuentes no costosas de carbohidratos fermentables. Recientemente también se vienen sumando esfuerzos en consolidar sistemas fermentativos con el fin de producir ácido láctico mediante la utilización de especies termofílicas como *Bacillus* sp., microorganismos que permiten el empleo de condiciones operacionales sin la necesidad de esterilidad (Gao et al., 2012; Meng et al., 2012; Ouyang et al., 2012; Patel et al., 2006).

Así, el presente trabajo se ha centrado en este último ámbito, estudiando la eficiencia del suero de yogurt como nueva y sostenible fuente de carbohidratos fermentables a través de una bacteria ácido-láctica con el objetivo de producir ácido L(+)-láctico. *L. casei* es una bacteria heterofermentativa facultativa que bajo las condiciones ensayadas ha metabolizado los correspondientes carbohidratos sin la consiguiente formación de ácido acético o etanol. Como se ha explicado *grosso modo* en el subapartado correspondiente (2.1), tanto los

requerimientos nutricionales como la productividad de un microorganismo acido-láctico en un proceso fermentativo están fuertemente influenciados por la naturaleza de la fuente de carbono presente en el medio. Así, las bacterias del ácido láctico utilizan los carbohidratos siguiendo de forma estricta un control jerárquico para el cual han desarrollado una serie de mecanismos de control que dirigen y coordinan la regulación específica del metabolismo central.

El consumo de carbohidratos por *L. casei* ha reflejado la capacidad de este microorganismo para metabolizar de forma eficiente las fuentes de carbono presentes en el suero de yogurt. Las Figuras 5.1 y 5.2 muestran la capacidad metabólica de *L. casei*. Estos resultados han sugerido un consumo co-metabólico llevado a cabo por *L. casei*, a partir del cual se puede inferir que este microorganismo es capaz de metabolizar activamente la glucosa, la sacarosa y la lactosa presentes en el medio. Sin embargo, hay un perfil de preferencia de consumo de estos azúcares, así, la glucosa es el primer azúcar metabolizado, mientras que el contenido en lactosa apenas se ve ligeramente reducido con el tiempo (Figura 5.2). Dicho contenido residual es similar en todos los casos, lo que sugiere que la mejora en el sistema operacional adoptado (control de pH y/o suplemento de extracto de levadura) no estimula el consumo de lactosa. Sin embargo, el control del pH y/o la presencia de extracto de levadura en precultivo sí han estimulado tanto el consumo de glucosa como de sacarosa (Figura 5.2).

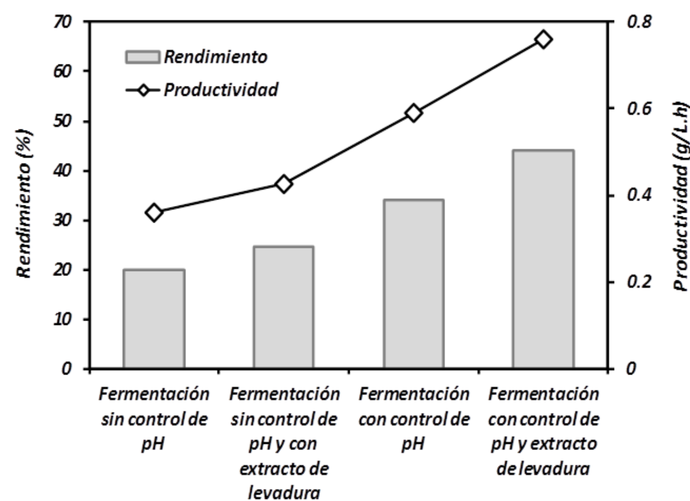


Figura 5.1. Rendimiento (porcentaje de carbohidratos consumidos sobre el contenido en azúcares totales) y productividad volumétrica de los distintos procesos fermentativos llevados a cabo para obtener ácido láctico.

Asimismo, estos resultados han demostrado que *L. casei* prefiere la glucosa y sacarosa sobre la lactosa como fuente primaria de carbono y energía a lo largo de un proceso ácido-

láctico. De hecho, el consumo de carbohidratos ha seguido un patrón muy similar en todos los casos, pero en cada caso, la cantidad producida de ácido láctico ha sido distinta debido al rendimiento diferencial de cada proceso fermentativo. La presencia de extracto de levadura en el precultivo junto con el control del pH incrementó el rendimiento hasta 0.9 g/g frente a valores similares entorno a 0.71 g/g encontrados sin control de pH. Como se muestra en la Figura 5.1, la productividad volumétrica alcanzó valores de 0.76 g/L.h bajo condiciones de control de pH y en presencia de extracto de levadura en el precultivo, siendo 2.1 veces superior a la productividad lograda sin control de pH (0.36 g/L.h). El consumo metabólico de estos carbohidratos fermentables ha seguido además una secuencia, algo que sugiere una utilización jerárquica y simultánea, llevada a cabo por un estricto mecanismo de control metabólico. A partir de los perfiles de consumo se puede inferir también que *L.casei* presenta diferentes rendimientos de ácido láctico producido en base al sustrato empleado, siendo más altas las relativas al consumo de glucosa y sacarosa (Figura 5.2).

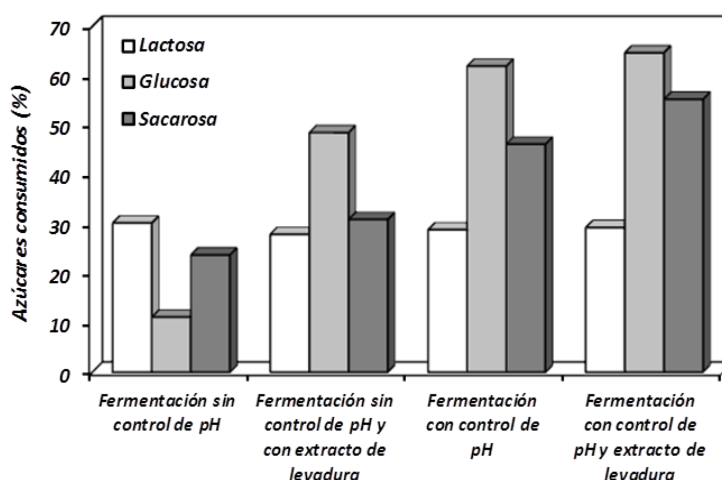


Figura 5.2. Porcentaje de carbohidratos consumidos tras 34 h respecto al contenido inicial.

En concreto, en *L. casei*, la lactosa es translocada al interior celular como lactosa fosfato, donde es hidrolizada en glucosa y galactosa-6-fosfato por una fosfo-β-D-galactosidasa. En función del modo de transporte, los disacáridos (caso de la sacarosa o la lactosa) pueden entrar en la célula bien en forma libre o fosforilada. En el primer caso, los disacáridos libres son hidrolizados en monosacáridos por hidrolasas específicas. La translocación como azúcar fosfato implica la presencia del sistema enzimático de carbohidrato fosfotransferasa, así como las fosfohidrolasas específicas que se encargan de hidrolizar el disacárido fosfato en dos monosacáridos, uno libre y otro con el grupo fosfato. Mientras que la glucosa es

fosforilada por una glucoquinasa y metabolizada a través de la vía glucolítica, la galactosa-6-fosfato es metabolizada a través de la ruta tagatosa-6-fosfato (Figura 5.3). Asimismo, la utilización de la sacarosa también está mediada por el sistema sacarosa fosfotransferasa. Una vez internalizado, el catabolismo de este disacárido comienza con la hidrólisis por la sacarosa-6-fosfato hidrolasa dando lugar a glucosa-6-fosfato y fructosa, las cuales entran en la vía glucolítica (Figura 5.3). Tanto el sistema sacarosa fosfotransferasa como la sacrosa-6-fosfato hidrolasa son inducidos por la presencia de sacarosa en el medio de cultivo. En el caso de la glucosa, ésta es translocada al interior celular en su forma fosforilada a través del sistema glucosa fosfotransferasa, siendo metabolizada posteriormente a través de la ruta catabólica Embden-Meyerhoff.

La glucolisis (conocida también como ruta Embden-Meyerhoff) se caracteriza por la formación de fructosa-1,6-difosfato que es hidrolizada por una aldolasa en dihidroxiacetona fosfato y gliceraldehído-3-fosfato. Este último metabolito es transformado en piruvato mediante la fosforilación a nivel de sustrato en dos sitios específicos distintos. De esta forma, el piruvato es reducido a ácido láctico por una lactato deshidrogenasa dependiente de NADH (el NADH formado en etapas previas se reoxida) bajo condiciones normales (microaerófilas). Con la obtención de este balance rédox metabólico se obtiene ácido láctico como producto final de la fermentación homoláctica. En el supuesto caso de que no exista un aceptor adicional de electrones, el acetilfosfato es reducido a etanol vía acetil-CoA y acetaldehído, dando lugar a la formación de etanol o ácido acético en la llamada fermentación heteroláctica (Panesar et al., 2007; Salminen et al., 2004).

L. casei presenta un patrón de consumo secuencial que no ha sido descrito con anterioridad para esta bacteria ácido láctica, siguiendo el siguiente orden: glucosa > sacarosa > lactosa. Así, *L. casei* presenta un co-consumo de glucosa, sacarosa y lactosa presentes en el suero de yogurt, siendo glucosa y la sacarosa los sustratos preferenciales. Los patrones de consumo, bien sean secuenciales, simultáneos o mixtos, son modificados en base a la presencia de diferentes carbohidratos. De hecho, se ha descrito cómo *Pediococcus pentosaceus* ha experimentado diferente patrón de consumo en función de los diferentes carbohidratos presentes en el medio de cultivo durante la producción de ácido láctico. Mientras que la galactosa y arabinosa fueron co-metabolizados, el resto de azúcares procedentes de residuos lignocelulósicos fueron metabolizados de forma secuencial (Adler et al., 2012).

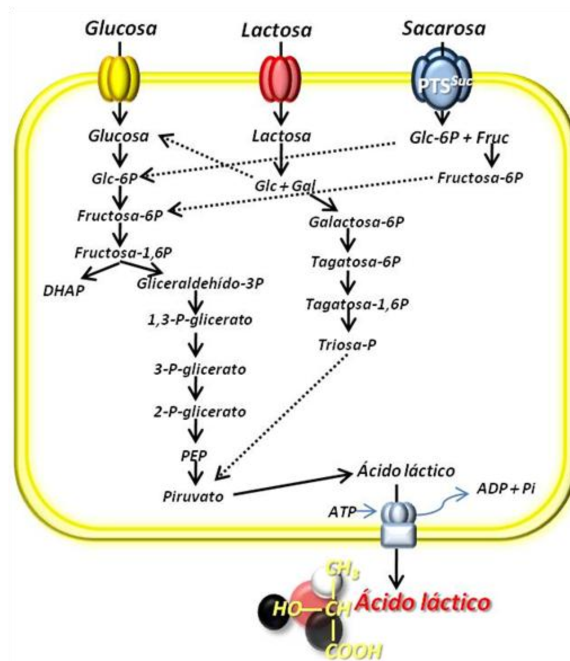


Figura 5.3. Rutas metabólicas involucradas en la producción de ácido láctico en *L. casei* a partir de glucosa, lactosa y sacarosa.

Los altos niveles de lactosa residual, presentes en el medio una vez terminada la fermentación, revelan la baja afinidad de *L. casei* por la lactosa en presencia de fuentes de carbono extras como representan la glucosa y la sacarosa. Estos resultados sugieren que este carbohidrato no comienza a ser consumido por *L. casei* hasta el agotamiento de las fuentes de carbono preferenciales, como son la glucosa y sacarosa. La baja tasa de asimilación de la lactosa se explica por la probable presencia de un sistema fosfotransferasa inducido con mayor afinidad por la glucosa y la sacarosa. No obstante, *L. casei* fue capaz de metabolizar la mayoría del contenido en glucosa y sacarosa en 48 horas consiguiendo rendimientos superiores al 80% de producción de ácido láctico y con valores superiores a 42 g/L en el caso de una fermentación sin suplemento de extracto de levadura en el preinóculo. El rendimiento (g/g consumido) de cada fermentación muestra cómo la mejora de las condiciones operacionales repercutió favorablemente sobre la producción de ácido láctico. Indudablemente, como anteriormente se ha resaltado, la mejora de las condiciones operacionales unidas a la presencia de extracto de levadura en el preinóculo logró mejorar el rendimiento global de la fermentación (42.54 versus 30.1 g/L de carbohidratos residuales).

En relación al status fisiológico, como se ha mostrado en el subapartado correspondiente (4.2), la fisiología de *L. casei* se ve fuertemente afectada por las diferentes condiciones microambientales. Es indudable la importancia del conocimiento preciso de la fisiología

poblacional de la bacteria empleada como plataforma biocatalítica en un sistema. La citometría de flujo multi-paramétrica permite la monitorización y el seguimiento de los bioprocesos microbianos, conociendo asimismo con exactitud las distintas propiedades celulares que influyen decisivamente sobre el status fisiológico poblacional (Díaz et al., 2010; Müller y Nebe-von-Caron, 2010; Tracy et al., 2010). Así, el análisis multi-paramétrico (enfocado al conocimiento de diferentes propiedades celulares) permite evaluar incluso el impacto de diversos factores sobre la propia célula como tal, evaluando incluso la posible toxicidad del metabolito producido o del propio sustrato (Figura 5.4). Esta caracterización a nivel poblacional del bioproceso permite realizar modificaciones precisas sobre el sistema con el fin de optimizar el proceso biocatalítico en términos de productividad y rendimiento global. Son múltiples los estudios que analizan y caracterizan diferentes bioprocesos mediante citometría de flujo, así esta técnica ha sido empleada con éxito en diferentes bioprocesos involucrados en bioconversiones microbianas (Amanullah et al., 2002a, 2002b, 2003) e incluso en fermentaciones de cerveza (Kobayashi et al., 2007) o de sidra (Herrero et al., 2006; Quirós et al., 2007).

Los conocimientos que aporta el empleo de esta técnica en estos sistemas microbianos van desde la cuantificación de la viabilidad celular a la caracterización del status fisiológico poblacional, dos parámetros que no pueden ser detectados ni cuantificados siguiendo técnicas convencionales. Asimismo, esta técnica está siendo empleada en procesos relacionados con la seguridad alimentaria con el fin de evaluar la eficacia de diferentes técnicas antimicrobianas tales como el empleo de altas presiones o fluidos supercríticos. Por tanto, a través del empleo de la citometría de flujo es posible monitorizar en tiempo real el estado metabólico-fisiológico de toda población microbiana involucrada en cualquier proceso de interés e incluso se presenta como una herramienta prometedora en la evaluación de diferentes fenotipos microbianos surgidos a consecuencia de la ingeniería metabólica.

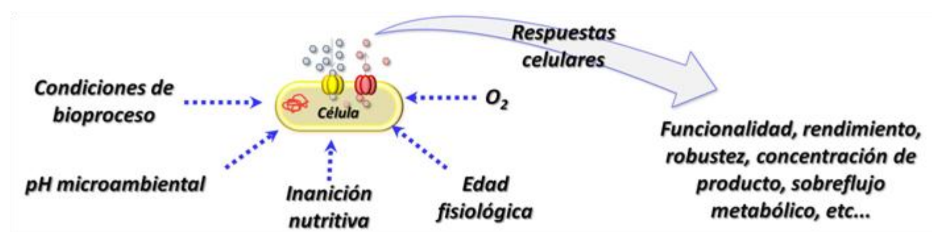


Figura 5.4. Impacto directo de diferentes variables microambientales sobre el microorganismo y cómo éste responde frente al stress con diferentes patrones de modulación fisiológica.

El protocolo de citometría de flujo empleado en el presente trabajo ha permitido evaluar el estado fisiológico de *L. casei* en relación a dos importantes parámetros como son la actividad metabólica y la integridad de membrana con el fin de elucidar y monitorizar la heterogeneidad subpoblacional en las diferentes fermentaciones. Igualmente, la utilización de un protocolo de citometría de flujo ha proporcionado una valiosa información acerca de la influencia del microambiente sobre la fisiología celular de *L. casei*. Los ensayos de viabilidad realizados en el presente trabajo se han llevado a cabo mediante tinción dual de las muestras con el fin de discriminar células con membranas intactas y con actividad enzimática (células metabólicamente activas) de las células dañadas y muertas (células con la membrana dañada). La pérdida de la integridad de membrana (ligada al daño en la membrana citoplásmica) implica la entrada en un estado fisiológico intermedio que puede sobrellevar con la muerte celular debido a la depleción energética celular (se disipa la fuerza protón motriz). Este estado fisiológico intermedio está constituido por subpoblaciones celulares que presentan una doble dualidad funcional, presentando tanto actividad enzimática como daños en la membrana. Por lo tanto, la evolución de un cultivo puede implicar que las células vivas y con estrés fisiológico entren en un estado fisiológico intermedio o en fase de muerte celular. Precisamente, un protocolo de citometría de flujo con tinción dual puede distinguir los diferentes patrones en los cambios de viabilidad celular dentro de un bioproceso como el que puede lleva a cabo *L. casei* en el lactosuero de yogurt. De esta forma, ha sido posible asimismo elucidar el papel que representa el suplemento con extracto de levadura sobre la viabilidad y la integridad de membrana.

La fermentación con control de pH se caracterizó por un progresivo cambio en el status fisiológico de *L. casei*, sugiriendo una importante influencia del nivel de lactato producido (~45 g/L) sobre la propia fisiología del microorganismo caracterizándose por un paulatino aumento en las subpoblaciones dañada y muerta. Similar fenómeno ha sido descrito por Rault et al. (2008) quienes han descrito la pérdida de la integridad de membrana de *L. delbrueckii* ante concentraciones altas de lactato durante la fermentación. Por el contrario, la falta de control de pH no influyo decisivamente sobre el status fisiológico de *L. casei* manteniendo un robusto status fisiológico a lo largo de la fermentación a pesar de la acidez (pH=3.5). Este patrón de comportamiento sugiere que *L. casei* exhibió una respuesta tolerante frente a la acidez bajo estas condiciones a lo largo de la fase estacionaria, en la cual no se detectó descenso alguno en la subpoblación metabólicamente activa. De hecho, se ha descrito que la bajada en el pH intracelular no fue acompañada por una pérdida de la viabilidad puesto que las bacterias ácido-lácticas desarrollan mecanismos de resistencia para evitar la influencia del pH microambiental sobre la fisiología. Recientemente, Wu et al.

(2012) han descrito el mantenimiento de la funcionalidad de la membrana plasmática de *L. casei* bajo condiciones de stress ácido. Así, *L. casei* desarrolla una respuesta tolerante al stress mediante la transcripción de diferentes familias genes que regulan dicha respuesta bajo estas condiciones microambientales (Broadbent et al., 2010). Asimismo, la presencia de azúcares adicionales bajo condiciones de inanición puede suponer un incremento en la viabilidad y/o mantenimiento de las funciones metabólicas de las bacterias ácido-láctica durante prolongados periodos de almacenamiento (Chen et al., 2011).

En definitiva, el desarrollo de este sistema ha logrado la reducción del impacto final de este residuo alimentario caracterizado por su alta concentración en carbohidratos fermentables, constituyendo hasta la fecha la única aproximación para el aprovechamiento biotecnológico de este residuo. De hecho, este residuo se ha mostrado como una matriz adecuada para la producción de ácido láctico sin la utilización de pretratamientos asociados a la sacarificación de residuos lignocelulósicos (John et al., 2009). A diferencia de otros sistemas desarrollados con otros sustratos, las fermentaciones discontinuas aquí desarrolladas fueron realizadas sin la adición de nutrientes extra durante el curso de las mismas, enfatizando en el bajo coste del sistema aquí desarrollado. Asimismo, el empleo de este residuo, una vez fermentado por *L. casei* y por lo tanto con altos niveles de ácido láctico, puede ser de particular interés en la industria de alimentación animal ya que el empleo de un suplemento alimentario con ácido láctico puede evitar las indeseables y recurrentes diarreas por enterobacterias en lechones o terneros.

5.2. *Producción biotecnológica de ácido lactobiónico*

Como se ha venido resaltando a lo largo de la presente memoria, las múltiples y novedosas aplicaciones del ácido lactobiónico en ámbitos tan diversos como el farmacéutico o el alimentario han estimulado la búsqueda activa de nuevas metodologías relacionadas con la producción del mismo. A diferencia de otros ácidos orgánicos como el succínico o el láctico, la obtención microbiana de ácido lactobiónico no ha sido estudiada de forma profusa hasta la fecha (en los subapartados 2.2 y 4.7 se pueden encontrar las comparaciones entre los diferentes sistemas biotecnológicos desarrollados hasta la fecha con el objetivo de producir este polihidroxi ácido). Indudablemente, uno de los objetivos fundamentales del presente trabajo ha sido aportar información valiosa ante la falta de conocimientos relativa a la producción biotecnológica de ácido lactobiónico. De igual forma, se ha intentado aportar nuevos conocimientos que puedan ayudar en el avance tecnológico de dicho ácido orgánico. Una síntesis de los principales hitos en la cronología histórica del ácido lactobiónico se muestra en la Figura 5.5.

La producción biotecnológica de ácido lactobiónico es una metodología que en comparación con la metodología tradicional (catálisis química) presenta múltiples ventajas, entre las que destaca la escasa formación de subproductos durante la reacción (empleando obviamente el microorganismo adecuado) a diferencia de muchos de los procesos de catálisis química donde la selectividad es inferior al 100%, lo que significa la formación paralela de subproductos durante el curso de la reacción.

A la hora de obtener ácido lactobiónico a escala de laboratorio, la catálisis enzimática representa la estrategia adoptada más ampliamente. Sin embargo, esta estrategia necesita la presencia de un adecuado aceptor de los protones liberados durante la oxidación (FAD). Además, también precisa de laboriosas etapas previas necesarias para la solubilización y purificación de los enzimas asociados a la membrana, caso de la oxidorreductosa de *Paraconiothyrium* sp. KD-3 (Kiryu et al., 2008). Satory et al. (1997) han obtenido ácido lactobiónico a través de una oxidación enzimática empleando como enzima a la glucosa-deshidrogenasa purificada a partir de *Zymomonas mobilis*. Al igual que muchos trabajos, no utilizan suero lácteo como sustrato en la bioconversión, otro inconveniente añadido es la formación de otros metabolitos (caso del sorbitol) como consecuencia del empleo de un enzima que presenta selectividad por diferentes sustratos. Estos investigadores han conseguido un 44% de conversión del sustrato inicial, pero por el contrario también obtienen una cantidad similar de sorbitol. Pedruzzi et al. (2011) también han utilizado una estrategia similar empleando células de *Z. mobilis* previamente permeabilizadas. De hecho,

las enzimas contenidas en las células permeabilizadas de *Z. mobilis* biotransforman mezclas de lactosa y fructosa, generando por lo tanto concentraciones equimolares de sorbitol como subproducto de la reacción (Malvessi et al., 2012; Pedruzzi et al., 2011).

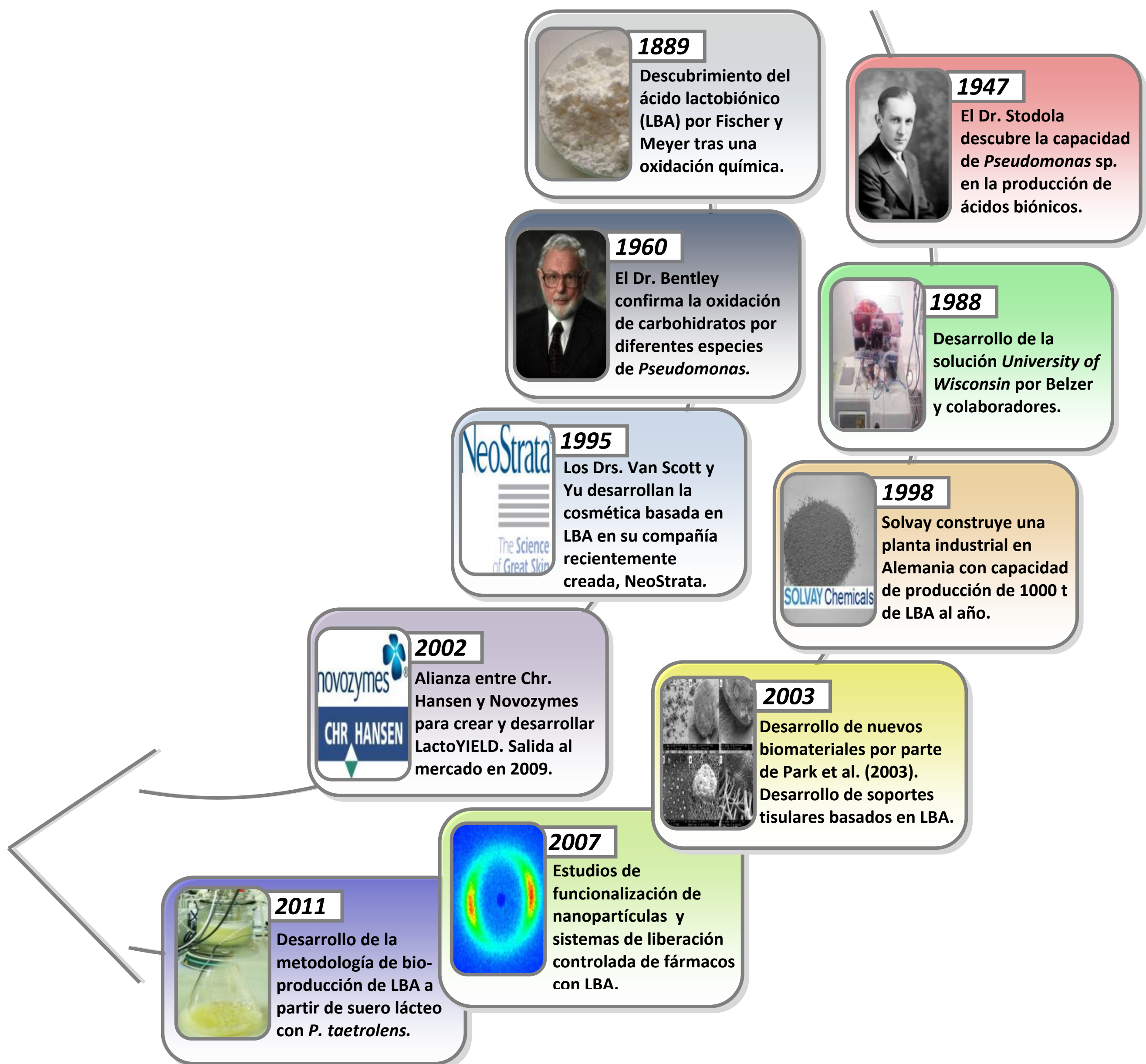


Figura 5.5. Cronología temporal e histórica del desarrollo del ácido lactobiónico. Principales hitos en la relativamente larga, intensa y a la vez desconocida historia del ácido lactobiónico.

Por lo tanto, la obtención biotecnológica del ácido lactobiónico surge como una alternativa más que adecuada ante los innumerables inconvenientes del resto de estrategias de producción. Sin embargo, hasta la fecha, en la bibliografía (véase subapartado 2.2) no se emplea suero lácteo como sustrato de la bioconversión. Así, Miyamoto et al. (2000)

emplearon para la producción de ácido lactobiónico 50 g/L de lactosa inicial sobre la que añadieron sales (KH_2PO_4 y $MgSO_4$) junto con una fuente de nitrógeno (peptona). Estos investigadores hallaron en sus investigaciones que la adición de 5-10 g/L de peptona como fuente de nitrógeno al medio de cultivo promueve significativamente la producción de ácido lactobiónico por parte de las cepas mutantes de *Pseudomonas* LS13-1, acortando además el proceso de transformación a 60 h. Además, estos investigadores lograron rendimientos en torno al 90% con una producción de hasta 290 g/L de ácido lactobiónico tras 155 h empleando una metodología de alimentación en discontinuo (Miyamoto et al., 2000).

La obtención de ácidos aldobionicos por *B. cepacia* también ha sido abordada por Meiberg et al. (1999). De hecho, han patentado un proceso biotecnológico con el fin de obtener ácidos biónicos (maltobiónico y lactobiónico) a partir de disacáridos reductores (lactosa y maltosa) utilizando como sustrato adicional sales minerales y diversas fuentes de nitrógeno. Sin embargo, en este proceso presenta el inconveniente de utilizar un microorganismo patógeno, así como el empleo de sustratos excesivamente complejos y realmente costosos. Murakami y colaboradores también han llegado a conseguir la bioconversion de 150 g/L de lactosa en ácido lactobiónico tras 24 horas de cultivo empleando células en reposo de mutantes de *B. cepacia* (resistente a altas concentraciones de lactosa) (Murakami et al., 2002, 2003, 2006).

P. taetrolens es una bacteria gram negativa aeróbica que se caracteriza por oxidar la lactosa para dar ácido lactobiónico sin hidrólisis del enlace glucosídico. Se trata de una especie originalmente aislada e identificada a partir de ovoproductos en proceso de putrefacción (Levine y Anderson 1931; West, 2004). La capacidad oxidativa del microorganismo fue descubierta a finales de los años 40 por Stodola y Lockwood (1947) como miembros de la división de fermentación perteneciente a los Laboratorios Regionales del Noreste (Illinois, USA). Estos investigadores llegaron en su momento a lograr un 77% de bioconversión de la lactosa inicial utilizando una metodología de cultivo en matraz agitado. De acuerdo con su origen, se puede aventurar que *P. taetrolens* es una bacteria que puede sobre-expresar genes relacionados con el deterioro de diversas matrices o sustratos como son los alimentos, y que asimismo posee la capacidad de oxidar determinados carbohidratos como elemento evolutivo que le permite competir con otras bacterias presentes en el mismo nicho. De esta forma se podría justificar la subida en los valores de pH en sustratos ricos en nitrógeno que surgen como resultado de la liberación al medio de cultivo de determinados compuestos básicos procedentes del metabolismo bacteriano.

La capacidad oxidativa de la lactosa llevada a cabo por *P. taetrolens* se ha puesto de manifiesto con un screening oxidativo previo realizado sobre medio sólido. De hecho, esta

metodología ha puesto de manifiesto que la capacidad oxidativa no es inmediata, surge tras el crecimiento de las colonias, tratándose, por lo tanto, de una capacidad asociada a la entrada en fase estacionaria (véase subapartado 4.3). Como se observa en estas bioconversiones, el ácido lactobiónico es un metabolito cuya producción por *P. taetrolens* no está asociado al crecimiento y sí a la entrada en fase estacionaria, ya que durante la fase activa de proliferación la bacteria no oxida la lactosa.

La influencia del tamaño de inóculo sobre el sistema de producción biotecnológica ha sido uno de los parámetros estudiados. Así, se ha constatado el efecto positivo del tamaño del inóculo sobre la producción de ácido lactobiónico para todas las bioconversiones llevadas a cabo, tanto a pequeña escala en matraz Erlenmeyer como a nivel de biorreactor (Figuras 5.6 y 5.7). Se ha constatado que un incremento en el tamaño de ínoculo indujo mayores valores asociados al rendimiento y productividad debido al anticipo de la entrada en fase de producción. Por el contrario, la tasa específica de crecimiento sí se ha visto claramente reducida ante un incremento en el tamaño del inóculo. Estos resultados sugieren que para tamaños de inóculo pequeños el margen existente para la proliferación celular es muy amplio al existir mayor ratio sustrato/biomasa (evidenciado además por un incremento significativo del valor máximo de salto del pH). Obviamente, el incremento en el tamaño de inóculo provoca una reducción de la tasa específica de crecimiento puesto que los requerimientos son mayores en términos de nutrientes y oxígeno disuelto. Así, para tamaños de inóculos grandes, llega un momento en el que estos requerimientos son mayores que la cantidad presente de nutrientes, reduciendo por tanto la tasa de crecimiento. Igualmente, la cantidad de micronutrientes presentes en el suero lácteo (vitaminas, sales minerales, aminoácidos, etc…) destinada al mantenimiento ha de ser superior, con lo cual el sustrato destinado a la proliferación/crecimiento se reduce.

Durante un cultivo microbiano son varios los parámetros que influyen decisivamente sobre la producción y rendimiento, se trata de parámetros que van desde la funcionalidad celular (status fisiológico) del microorganismo, la cantidad de oxígeno disuelto en el medio (influenciado por la agitación y la aireación), el pH o la riqueza de nutrientes del medio. El aporte de oxígeno disuelto juega un papel decisivo en el sistema como se ha puesto de manifiesto en el subapartado 4.4, ya que provee al microorganismo/sistema con la adecuada demanda de oxígeno y más si se trata de un proceso oxidativo como el que se estudia en parte de la presente memoria. Además, los estudios preliminares en matraz mostraron que el sistema está limitado por la falta de aireación durante el proceso. De hecho, el aporte de oxígeno es a menudo el factor limitante del crecimiento celular en matraz, ya que no se

incrementa la superficie de intercambio gaseoso y, en consecuencia, no se favorece la transferencia de materia.

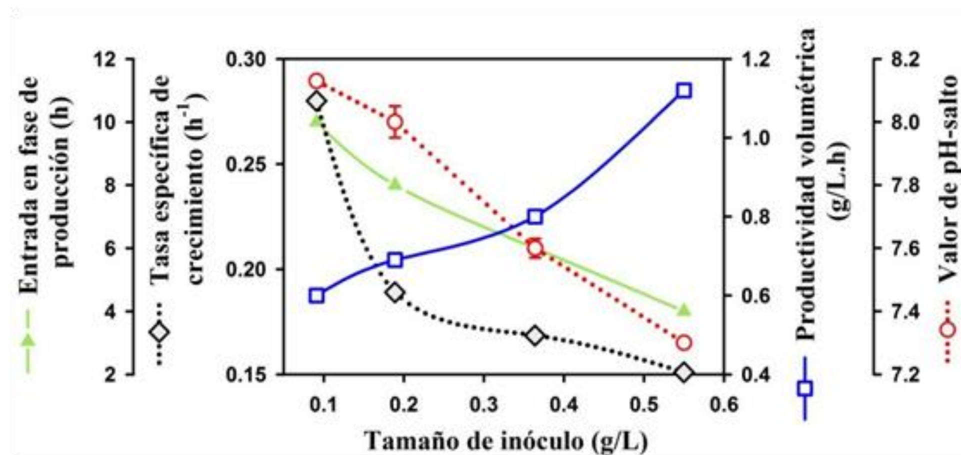


Figura 5.6. Relación entre el tamaño de inóculo y los diferentes parámetros del bioproceso a nivel de matraz.

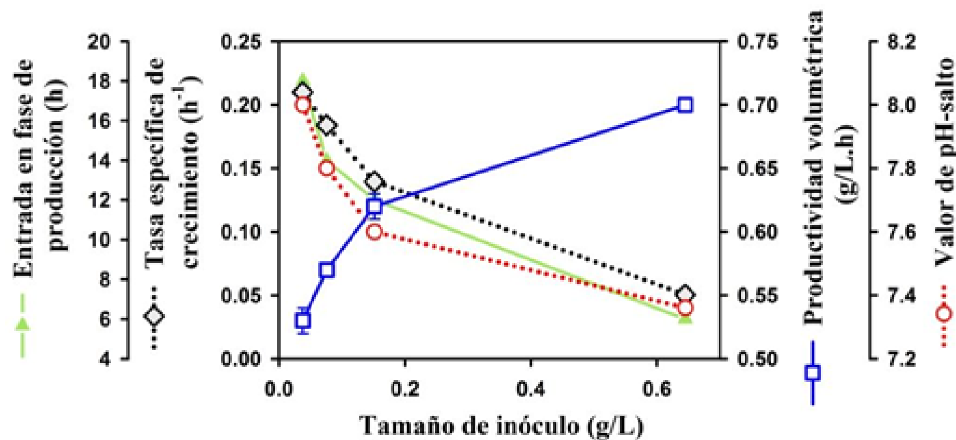


Figura 5.7. Relación entre el tamaño de inóculo y los diferentes parámetros obtenidos del bioproceso tras el cultivo en biorreactor.

Evidentemente, la concentración de oxigeno disuelto es un parámetro que cambia hasta 10 veces más rápido que la biomasa y la concentración de los sustratos. Este hecho sitúa al aporte de oxígeno disuelto como la variable más importante a la hora de controlar y optimizar bioproceso aeróbico como el aquí descrito. Por lo tanto, y dado que el pH también es un parámetro que influye decisivamente en el proceso, se ha procedido a implementar la bioconversión en biorreactor a diferentes niveles de agitación y aireación, además de regular el pH del cultivo a 6,5 para evitar los posibles efectos inhibitorios por parte de la acidez generada.

La constatación de que la disponibilidad de oxígeno disuelto constituye un parámetro influyente en el bioproceso se ha puesto de manifiesto con la puesta en marcha de un cultivo en iguales condiciones experimentales pero sin aireación. Así, como se ha reflejado en el subapartado 4.4, en apenas 20 minutos se agota todo el oxígeno disuelto en el medio, una circunstancia que impide la proliferación celular en los parámetros habituales a pesar de la existencia de un leve crecimiento (véase subapartado 4.4). No se observa el habitual incremento del pH durante el crecimiento en fase exponencial aún a pesar de la existencia de un leve crecimiento. A la vista de la evolución del cultivo y de las condiciones (anoxia completa), como cabría esperar los valores de producción de ácido lactobiónico y oxidación de la lactosa no se modificaron a lo largo del mismo.

Tasas de agitación superiores a 350 rpm favorecen la formación de biomasa, mientras que la producción de ácido lactobiónico se ve claramente mermada. Sorpresivamente esta tendencia observada bajo altas condiciones de aporte de oxígeno a través de la agitación, no se ha observado bajo tasas de aireación superiores a 1.5 Lpm. De hecho, un incremento en la aireación no se tradujo en un incremento en la proliferación celular. De las distintas variables microambientales estudiadas, se puede concluir que tanto el pH como la disponibilidad de oxígeno disuelto juegan un papel decisivo sobre la buena evolución del cultivo. Es importante tener en cuenta que la tasa de transferencia de oxígeno (OTR) siempre se ha mantenido por encima de la demanda de oxígeno (OUR) como se observa en la Figura 5.8.

Por lo tanto, se puede concluir que la adopción de estrategias operacionales con altos niveles de agitación (500-1000 rpm) estimula el crecimiento y la proliferación celular, pero por el contrario reduce la producción de ácido lactobiónico. Así, niveles medios de agitación (350-500 rpm) y aeración (0.5-1.0 Lpm) se mostraron como los más idóneos desde el punto de vista productivo (subapartado 4.4). Se ha comprobado igualmente que la presencia de un periodo corto de disponibilidad de oxígeno limitado, favorece y estimula los parámetros asociados a la producción de ácido lactobiónico (tanto el rendimiento, la entrada en fase de producción como la productividad volumétrica). De hecho, la introducción de una fase de microaerobiosis al final de la fase de crecimiento exponencial puede mejorar el rendimiento, concentración y productividad dentro de determinados sistemas (Martínez et al., 2010; Wieschalka et al., 2012). Es indudable que la ausencia total de aporte de oxígeno, por el contrario, restringe tanto la proliferación celular como la producción de ácido lactobiónico. La falta de oxígeno disuelto en el medio oxígeno inhibe por tanto la cadena respiratoria, impidiendo tanto la generación de ATP como la oxidación del cofactor $FADH_2$

involucrado en la deshidrogenación de la lactosa para formar ácido lactobiónico (véase subapartado 2.2).

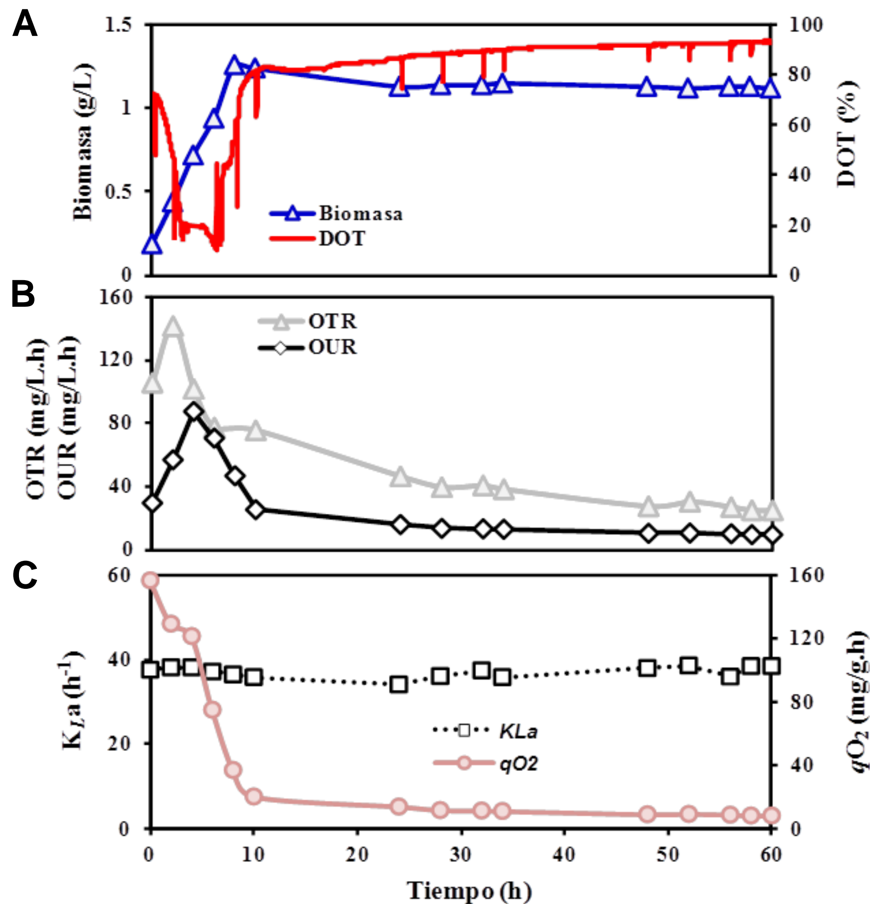


Figura 5.8. Comparación de los perfiles generados de biomasa y oxígeno disuelto (A), junto con los valores del aporte de oxígeno disuelto y consumo netos (B), así como los coeficientes volumétricos de transferencia de materia y el consumo específico (C) generados a lo largo de un bioproceso con una agitación de 350 rpm y una tasa de aireación de 1 Lpm.

La industria biotecnológica ha reconocido de hecho la importancia de un detallado conocimiento y caracterización de todo bioproceso asociado a la producción de metabolitos de interés. Muchos de los bioprocesos están asociados a una heterogeneidad fisiológica que puede constituir una verdadera limitación para el correcto desarrollo de un sistema asociado a la producción de metabolitos de valor añadido (Lara et al., 2006). De hecho, las aproximaciones reduccionistas representadas por las técnicas dependientes de cultivo reflejan una visión reducida del status fisiológico de la biomasa celular (Hewitt y Nebe-von-Caron, 2004) en contraposición con nuevas técnicas que permite conocer con exactitud el estado *saludable* (*healthy*) de los microorganismos como la citometría de flujo multi-paramétrica.

La evaluación a través de citometría de flujo del sistema biotecnológico aquí descrito, ha revelado que tanto la heterogeneidad poblacional como la funcionalidad de las células de *P. taetrolens* se ven claramente afectadas como respuesta a diferentes estímulos estresantes. Es indudable que cambios microambientales, tales como la inanición o el aporte excesivo de nutrientes externos pueden causar respuestas globales a nivel poblacional.

El estado fisiológico de una población bacteriana es clave a la hora de conseguir altas eficiencias y rendimientos. Por lo tanto, es clave detectar la presencia de células doblemente teñidas al inicio de un proceso de bioconversión, algo que se ha puesto de manifiesto con imágenes obtenidas con microscopio láser confocal de células de *P. taetrolens* durante el transcurso del proceso. Estos resultados demuestran la importancia del estado fisiológico celular en el comportamiento general dentro del bioproceso. Así, la presencia de fases lag sugiere un pobre status fisiológico intrínseco a los cultivos utilizados como inóculos de los cultivos realizados en biorreactor. De hecho el predominio de células dañadas y muertas en la fermentación inoculada con cultivos que presentan una edad fisiológica superior a 12-h redujo drásticamente la productividad del sistema, obteniendo resultados pobres en comparación con cultivos óptimos desde el punto de vista fisiológico (edad fisiológica de 12-h). Macroscópicamente, la presencia de fases lag indica una mala adaptación del microorganismo a las nuevas condiciones de cultivo. Descifrar la heterogeneidad fisiológica subyacente asociada a inóculos con edad superior a 12-h ha permitido, en este caso, optimizar y corregir los posibles errores operacionales que de otro modo serían inevitables y para los cuales no habría una explicación coherente. La sobre-exposición de estos precultivos a unas condiciones deletéreas ($pH<3.4$ a partir de 24 h) ha inducido una respuesta fisiológica frente al stress ácido (Figura 5.9). Dichos datos fueron corroborados igualmente a través de la visualización en microscopía de fluorescencia y a través de una separación celular (*fluorescence-activated cell sorting*, FACS) y posterior cultivo en medio sólido. De los resultados obtenidos, se puede inferir un 75% de las células dañadas (correspondientes a un cultivo de 24-h) se corresponden con células viables no cultivables (VBNC) (subapartado 4.5). A nivel celular, esta pérdida de viabilidad está asociada a respuestas funcionales y estructurales con cambios irreversibles en la estructura de la membrana o en la polarización de la misma (Figura 5.10). En definitiva, la heterogeneidad fisiológica a nivel poblacional ha provocado una reducción en la funcionalidad celular, con la consiguiente reducción en el rendimiento del bioproceso (Figura 5.11).

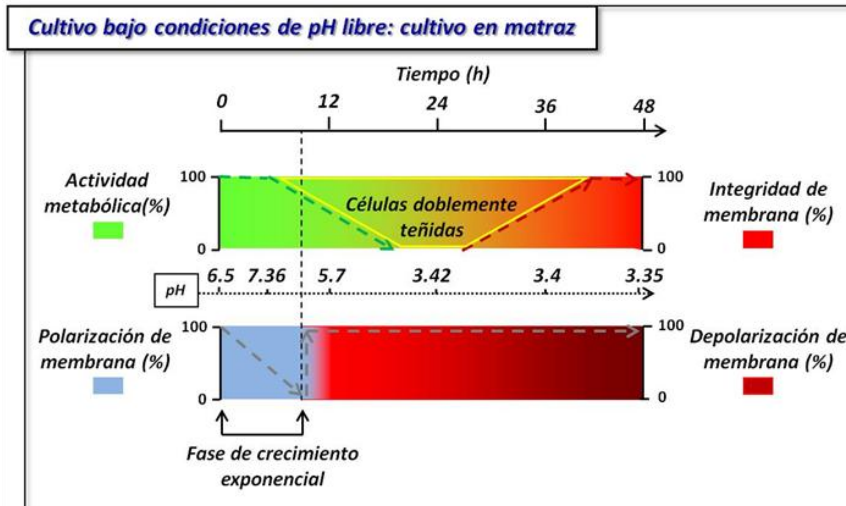


Figura 5.9. Diagrama que muestra *grosso modo* los cambios fisiológicos y estructurales en las células de *P. taetrolens* bajo condiciones de pH libre. Como se observa, los cambios se asocian en primer lugar a una bajada drástica en el pH tras la fase exponencial y a la posterior sobre-exposición bajo condiciones deletéreas (pH~3,4) ya en fase estacionaria tardía (>24 h).

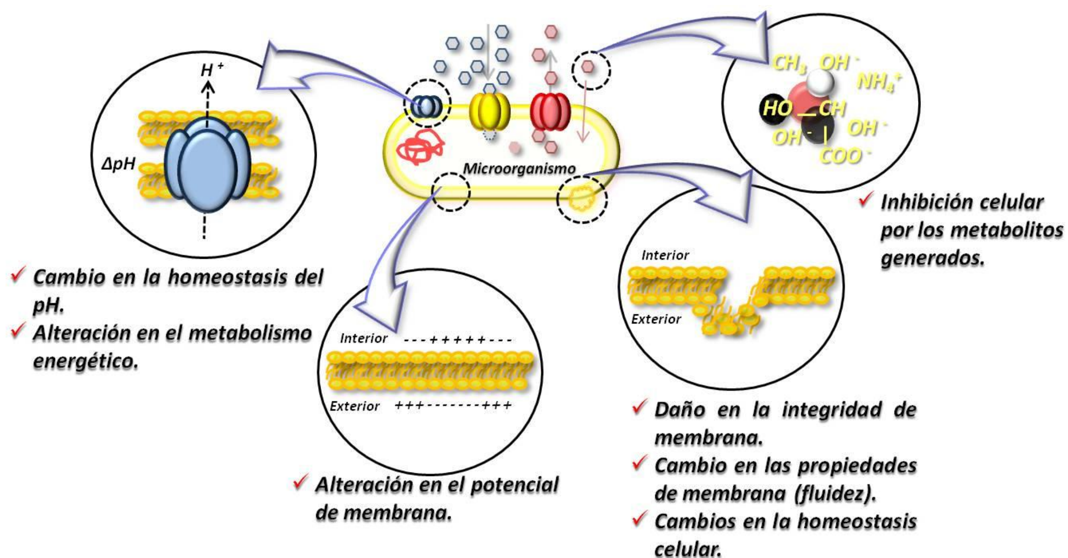


Figura 5.10. Daños funcionales y estructurales generados a nivel celular como respuesta frente a estímulos estresantes.

El inadecuado pH microambiental ha provocado a su vez una respuesta inducida frente al stress. De esta forma se ha podido conocer el origen de una reducción en los parámetros asociados a la producción bajo diferentes condiciones operacionales como son el pH inicial (Figura 5.12), el pH controlado en fase de producción (Figura 5.13) o durante todo el proceso (Figura 5.14). En suma, el status funcional y la heterogeneidad fisiológica de *P.*

taetrolens son desde luego dos parámetros fundamentales y críticos en la producción de ácido lactobiónico a partir de suero lácteo.

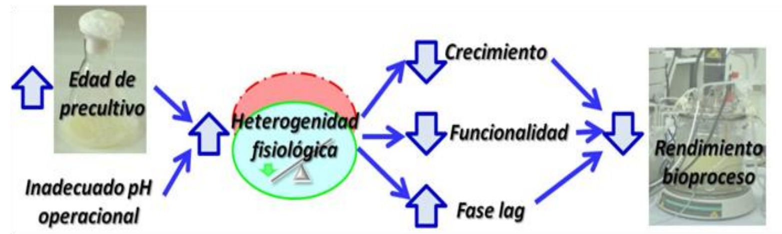


Figura 5.11. Relación directa entre la heterogeneidad fisiológica con el inadecuado pH operacional y la edad fisiológica del inóculo.

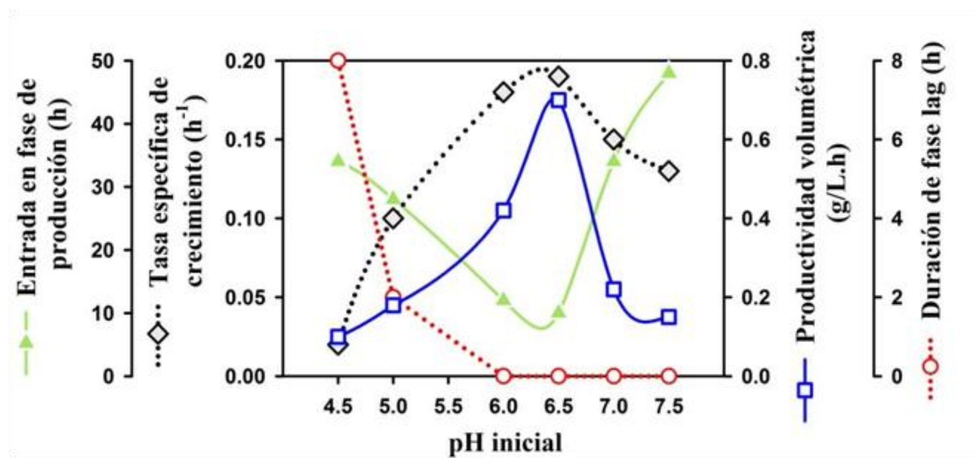


Figura 5.12. Relación entre el pH inicial y los diferentes parámetros del bioproceso.

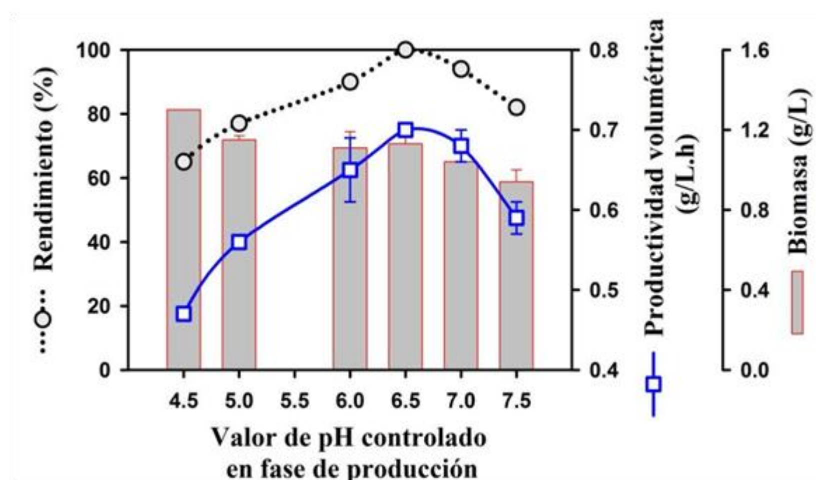


Figura 5.13. Relación entre el valor de pH controlado en fase de producción (pH-shift) y los diferentes parámetros del bioproceso.

Tanto valores de pH inicial inferiores como superiores a 6,5 llevan consigo una reducción en la productividad volumétrica, así como un incremento en la entrada en fase de producción. Igualmente, la tasa específica de crecimiento ha encontrado en 6,5 el valor óptimo de pH inicial (Figura 5.12).

En relación al valor más adecuado de pH controlado en fase de producción, se ha constatado que 6,5 es el valor óptimo de operación en términos de productividad volumétrica y rendimiento, a pesar de un incremento en la biomasa mantenida bajo valores inferiores a 6,0 (Figura 5.13). Valores de pH controlado de 6,0-6,5 fueron encontrados como los óptimos, así el empleo de valores fuera de este rango ha significado una reducción en la tasa específica de crecimiento, la aparición de fases lag o un retraso en la entrada en fase de producción (Figura 5.14).

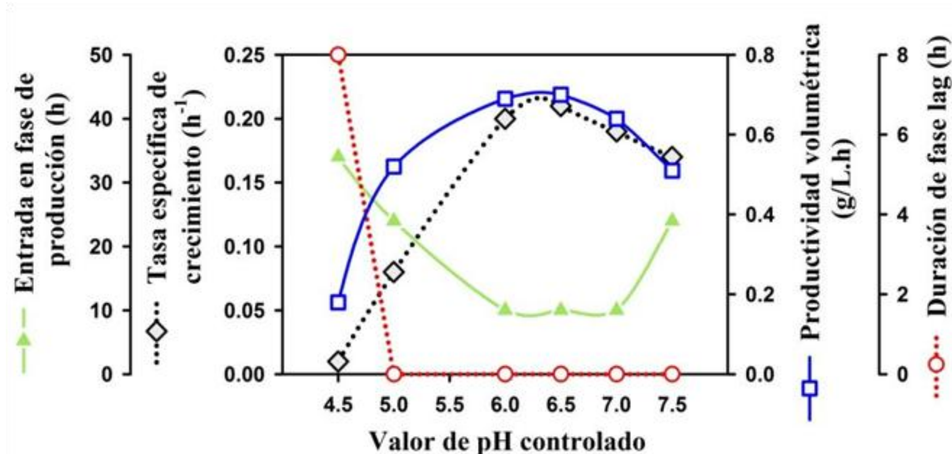
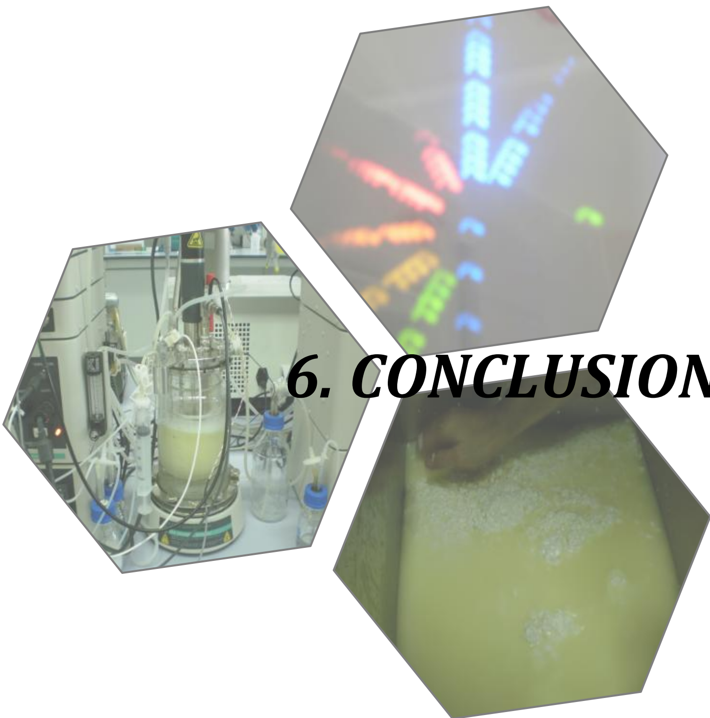


Figura 5.14. Relación entre el valor de pH controlado (pH-stat) y los diferentes parámetros del bioproceso.

Tras la optimización de las diferentes variables, tanto el incremento de la productividad volumétrica, así como la concentración producida de ácido lactobónico se han convertido en los objetivos principales de la presente memoria. Por lo tanto, se ha procedido a incrementar estos valores asociados a la producción a través de un cultivo discontínuo con alimentación. Desde el desarrollo del cultivo en discontinuo con alimentación por Davey y Johnson en 1953 para la producción de penicilina, esta estrategia ha sido ampliamente adoptada por la industria como la solución para extender la fase de crecimiento/producción con el fin de obtener mejoras en el rendimiento de sistemas centrados en la obtención vía fermentativa de bio-productos tan diversos como ácidos orgánicos (Ding y Tan, 2006; Zhu et al., 2011), antibióticos (Müller et al., 2012) o proteínas recombinantes tales como la eritropoyetina (Soyaslan y Çalik, 2011). Se trata en definitiva de una metodología que

permite mejorar los parámetros de producción asociados a todo bioproceso. De hecho, conocer la influencia de diferentes estrategias de alimentación sobre el sistema puede llevar a la adopción de las estrategias más rentables desde el punto de vista productivo. Así, tasas de alimentación superiores a 8.4 mL/h redujeron drásticamente el rendimiento y productividad del sistema (subapartado 4.7). En este trabajo se ha desarrollado adicionalmente una metodología eficiente y sencilla de co-alimentación que ha logrado incrementar la concentración de lactosa disponible para la oxidación por parte de *P. taetrolens*. Además, dicha estrategia ha logrado mantener la funcionalidad celular de *P. taetrolens* a lo largo del proceso mediante el continuo aporte de nutrientes a tasas bajas. En definitiva, se ha propuesto una eficiente alternativa de producción biotecnológica de ácido lactobiónico que cumple los requisitos en cuanto a niveles de producción (80-100 g/L) se refiere, que la industria considera como necesarios para proponer a cualquier sistema biocatalítico de producción como alternativa factible a los sistemas basados en catálisis química (Pollard y Woodley, 2007; Yang et al., 2007).



6. CONCLUSIONES

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El trabajo anteriormente expuesto permite inferir las siguientes conclusiones:

- ✓ El suero lácteo de yogurt se ha revelado como una adecuada fuente para la producción de ácido láctico por *L. casei* a partir de una materia prima hasta ahora considerada como un residuo y que se caracteriza por presentar altos niveles de azúcares fermentables sin la necesidad de realizar pretratamiento alguno.

- ✓ *L. casei* metaboliza de forma jerárquica los azúcares presentes en el lactosuero de yogurt. Los rendimientos y balances fermentativos han mostrado un consumo simultáneo de la glucosa, lactosa y sacarosa. La evolución del contenido en azúcares ha puesto de manifiesto que la glucosa y la sacarosa son metabolizadas de forma más eficientemente, actuando como sustratos preferenciales en el catabolismo de *L. casei* bajo las condiciones de cultivo ensayadas. Por el contrario, la mejora y control de las condiciones operacionales no ha logrado incrementar la tasa de bioconversión de la lactosa durante el proceso fermentativo. A pesar de la modificación de las condiciones operacionales y de la presencia de extracto de levadura durante la fermentación, no se han producido cambios en el patrón fermentativo homoláctico de *L. casei*. A diferencia del resto de carbohidratos, el ratio de consumo de lactosa no se ha visto afectado negativamente por la presencia de un ambientes más estresante, menos controlado y pobre en nutrientes como es el caso de un proceso fermentativo con pH libre y sin suplementación con extracto de levadura.

- ✓ Los parámetros operacionales y la metodología empleada durante la fermentación acido láctica han influido decisivamente sobre el rendimiento productivo. De esta forma, la producción de ácido láctico se ha visto afectada por la presencia de extracto de levadura en el precultivo empleado como inóculo del proceso fermentativo final. Asimismo, la regulación del pH y la presencia de nutrientes extra han favorecido un incremento significativo en la cantidad de carbohidratos metabolizados por parte de *L. casei*, quien presenta un consumo simultáneo y a la vez preferencial de los mismos en el siguiente orden glucosa > sacarosa > lactosa.

- ✓ La monitorización del estado fisiológico de *L. casei* durante las fermentaciones ha permitido establecer y determinar la presencia simultánea de hasta tres

subpoblaciones distintas. Así, el incremento en la concentración de lactato encontrado bajo condiciones de control de pH ha podido ser asociado con una pérdida del status fisiológico de *L. casei*, mientras que un sistema sin control de pH (pH~3.5) ha logrado mantener el nivel inicial de funcionalidad a lo largo de toda la fermentación, sugiriendo una respuesta tolerante frente al stress por parte de *L. casei*.

- ✓ El suero lácteo se ha revelado como una matriz adecuada para la producción biotecnológica de ácido lactobiónico por parte de *P. taetrolens*, sugiriendo que este microorganismo puede llevar a cabo una bioconversión oxidativa de la lactosa de forma eficiente y competitiva. Asimismo, se ha descrito por primera vez cómo el proceso de formación de ácido lactobiónico no está asociado al crecimiento. Mientras que la fase de crecimiento exponencial de *P. taetrolens* se ha caracterizado por un abrupto ascenso del pH del medio y por un agotamiento en el contenido del oxígeno disuelto en el medio.

- ✓ Los datos obtenidos de la bioconversión a pequeña escala y en biorreactor han mostrado que la eficiencia, el rendimiento y la productividad del bioproceso han estado íntimamente ligados al tamaño de inóculo empleado. Así, una mayor cantidad de biomasa al inicio del proceso logra mejores rendimientos en la bioconversión tras promover la entrada en fase de producción bajo densidades celulares superiores, consiguiendo asimismo una significativa reducción de la duración de la fermentación.

- ✓ El papel del oxígeno disuelto se ha mostrado como determinante sobre el rendimiento y la eficacia del bioproceso. Así, la disponibilidad plena de oxígeno disuelto en el medio, bien a través de altas tasas de aireación (>1.5 Lpm) y/o agitación (>500 rpm), ha implicado una reducción importante en los valores producidos de ácido lactobiónico. Valores medios de aporte de oxígeno (350-500 rpm o 0,5-1 Lpm) se han mostrado como los óptimos en términos productivos.

- ✓ El sistema de producción de ácido lactobiónico por *P. taetrolens* lleva asociado una heterogeneidad fisiológica subyacente que afecta decisivamente al rendimiento productivo del sistema. Edades fisiológicas del inóculo superiores a 12-h reducen

drásticamente la producción de ácido lactobiónico debido al predominio de células dañadas y muertas durante el cultivo.

- ✓ La falta de control de pH (<4) unida al agotamiento en el contenido en oxígeno disuelto (<1%) encontrado en matraz agitado ha llevado a la inducción de una respuesta fisiológica frente al stress en *P. taetrolens*, caracterizada por la entrada progresiva e irreversible en muerte celular. Esta heterogeneidad fisiológica, caracterizada por la presencia de células dañadas y muertas, se ha mostrado como clave en el correcto progreso del sistema productivo. Así, la presencia de células dañadas y muertas ha implicado una reducción drástica en los valores asociados a la producción de ácido lactobiónico.

- ✓ Se ha logrado determinar el valor de pH operacional óptimo con el fin de maximizar el rendimiento del bioproceso. Así, estrategias con control de pH estricto (pH-stat) o bien sólo en fase de producción (pH-shift) a 6.5, son las más adecuadas tanto desde el punto de vista fisiológico como productivo. Igualmente, un valor de 6.5 como pH inicial se ha mostrado como el más adecuado, puesto que valores inferiores a 5.5 inducen la entrada en fase de producción sin la necesaria etapa previa de crecimiento.

- ✓ Se han alcanzado por primera vez niveles realmente atractivos para la industria biotecnológica tanto en la concentración (180 g/L) como en el rendimiento (>90%), empleando un residuo con escaso valor como sustrato en un sistema llevado a cabo por un microorganismo no recombinante.

7. BIBLIOGRAFÍA

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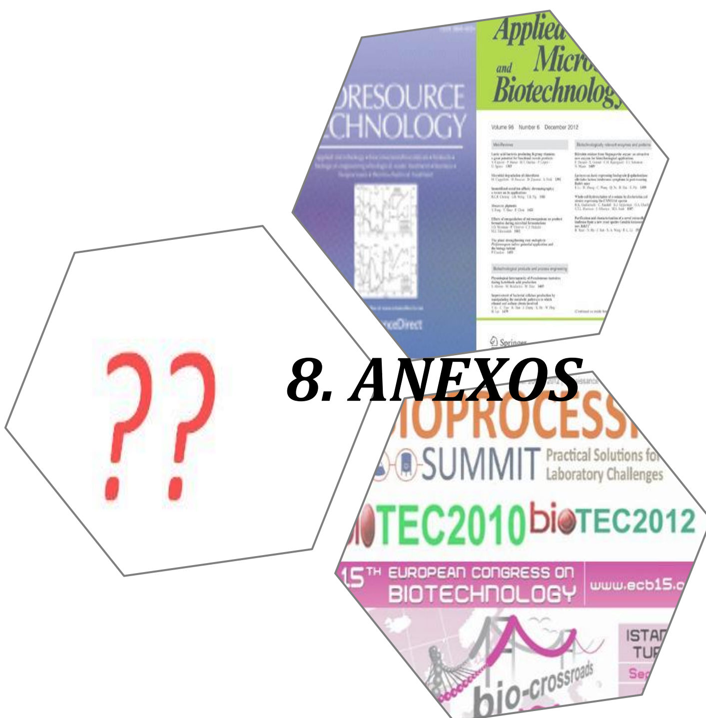
8. ANEXOS



8. ANEXOS

8.1. Difusión de la tesis doctoral

8.1.1. Artículos científicos

- **Alonso**, **S.**, Herrero, M., Rendueles, M., Díaz, M. 2010. Residual yoghurt whey for lactic acid production. *Biomass and Bioenergy* 34, 931-938.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. 2011. Efficient lactobionic acid production from whey by *Pseudomonas taetrolens* under pH-shift conditions. *Bioresource Technology* 102, 9730-9736.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. 2012. Role of dissolved oxygen availability on lactobionic acid production from whey by *Pseudomonas taetrolens*. *Bioresource Technology* 109, 140-147.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. 2012. Physiological heterogeneity of *Pseudomonas taetrolens* during lactobionic acid production. *Applied Microbiology and Biotechnology* 96, 1465-1477.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. 2012. Selection method of pH conditions to establish *Pseudomonas taetrolens* physiological states and lactobionic acid production. *Applied Microbiology and Biotechnology.* Aceptada, en prensa. doi: 10.1007/s00253-012-4607-x.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. Feeding strategies for enhanced lactobionic acid production from whey by *Pseudomonas taetrolens*. *Bioresource Technology.* Aceptada.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. Bio-production of lactobionic acid: current status, applications and future prospects. Enviada para su evaluación, bajo revisión.

8.1.2. *Comunicaciones a congresos*

- **Alonso**, **S.**, Herrero, M., Rendueles, M., Díaz, M. Whey fermentative processes with industrial interest (póster).11th Mediterranean Congress of Chemical Engineering. Barcelona (España). Octubre 21-24, 2008.

- **Alonso**, **S.**, Herrero, M., Rendueles, M., Díaz, M. Monitorization of physiological heterogeneity in a sugars-to-lactic bioprocess (comunicación oral, galardonada con el 3er premio como mejor comunicación oral). 5th International Meeting on Biotechnology BioSpain 2010. Pamplona (España). Septiembre 29-Octubre 1, 2010.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. Analysis of physiological states for bioreactor scale-up (póster). The Bioprocessing Summit 2012. Boston (Estados Unidos). Agosto 20-23, 2012.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. Application of multi-parameter flow cytometry to study the bioproduction of lactobionic acid (comunicación oral). 6th International Meeting on Biotechnology BioSpain 2012. Bilbao (España). Septiembre 19-21, 2012.

- **Alonso**, **S.**, Viña, J., Rendueles, M., Díaz, M. Effect of initial pH on shake-flask cultivation of *Pseudomonas taetrolens* (póster). 6th International Meeting on Biotechnology BioSpain 2012. Bilbao (España). Septiembre 19-21, 2012.

- **Alonso**, **S.**, Rendueles, M., Díaz, M. Physiological states of *Pseudomonas taetrolens* cultured in whey media under different oxygen and pH conditions (póster). 15th European Congress on Biotechnology. Estambul (Turquía). Septiembre 23-26, 2012.

8.2. *Informe sobre el índice de impacto de los artículos de la tesis*

Los artículos que conforman la presente memoria han sido publicados en revistas incluidas en el *Science Citation Index* (Thomson Reuters), cuyos índices de impacto son los siguientes:

Biomass and Bioenergy (2010) → 3.84

Bioresource Technology (2011) → 4.98

Applied Microbiology and Biotechnology (2011) → 3.42