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**“Modelado, simulación y análisis de procesos de producción de
biocombustibles por bacterias del género *Clostridium* bajo diferentes
esquemas de producción.”**

T E S I S

Que presenta

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Resumen

La fermentación ABE es un proceso biotecnológico que puede ser explotado para la generación de butanol como biocombustible, sin embargo, se necesita para superar su baja productividad volumétrica y concentración final de producto antes de que pueda competir comercialmente con los combustibles fósiles. En este sentido, las técnicas de optimización numérica aplicada sobre modelos matemáticos representativos del proceso de fermentación ABE son herramientas con las que se pueden desarrollar avances en la ingeniería de procesos aplicada a este sistema biológico.

En este trabajo un modelo cinético no estructurado de *Clostridium acetobutylicum* ATCC 824 validado con datos experimentales bajo diferentes condiciones de cultivo se utilizó para determinar las condiciones óptimas de proceso que maximizaran dos objetivos conflictivos: la productividad volumétrica de butanol y la concentración final del mismo considerando los siguientes regímenes de operación: lote con suplementación de ácido butírico, Fed-Batch y CSTR de una sola etapa, mediante la construcción de los Frentes de Pareto correspondientes con la metodología de Programación Cuadrática Secuencial. Los resultados obtenidos sugieren que la máxima concentración y productividad de butanol se consiguen en un sistema semi-continuo operando con células inmovilizadas, obteniendo valores de 16.89 g L⁻¹ y 0.4000 g L⁻¹ h⁻¹, respectivamente, lo que representa un incremento del 30.4247 % y del 122.23 % en comparación con el proceso industrial más reciente reportado hasta la fecha (Jiang et al., 2015).

Abstract

ABE fermentation is a biotechnological process that can be exploited for the generation of butanol as biofuel, however it does need to overcome its low volumetric productivity and final product concentration before it can compete commercially with fossil fuels. In this sense, numerical optimization techniques applied over representative mathematical models of the ABE fermentation process are tools with the potential to achieve promising advances towards the process engineering applied to this biological system.

In this work, an unstructured kinetic model of *Clostridium acetobutylicum* ATCC 824 validated with experimental data under different culture conditions was used to determine the optimal process conditions that would maximize two conflicting objectives: volumetric productivity and final concentration of butanol, considering the following operation regimes: batch with butyric acid supplementation, Fed-Batch and single-stage CSTR, by constructing the Pareto Fronts by the use of the Sequential Quadratic Programming methodology. The results obtained suggest that the maximum concentration and productivity of butanol are achieved in a semi-continuous system operating with immobilized cells, obtaining values of 16.89 g L^{-1} and $0.4000 \text{ g L}^{-1} \text{ h}^{-1}$, respectively, representing an increase of 30.4247 % and 122.23 % compared to the most recent industrial process reported to date (Jiang et al., 2015).

Introducción

Es bien sabido que la humanidad está experimentando en este momento una gran crisis energética porque la fuente principal proviene de un recurso no renovable como el petróleo. La realidad del sector petrolero indica que las reservas se encuentran en un nivel crítico, por lo que ahora el esfuerzo de investigación se centra en el estudio y desarrollo de procesos destinados a consolidar la producción de energía alternativa y la tecnología de distribución (Demirbas, 2009).

Uno de los objetivos clave del enfoque del uso de tecnologías energéticas alternativas es reducir el impacto ambiental causado por la explotación y el uso de la energía de combustibles fósiles, especialmente teniendo en cuenta el gran daño ambiental causado por la extracción, refinación y uso de derivados del petróleo, que son una amenaza tanto para los ecosistemas terrestres como marinos debido a la fuga de hidrocarburos aromáticos o moléculas de alto peso porque son inmiscibles en el agua y sus propiedades fisicoquímicas les otorgan una resistencia adicional en el ambiente donde se descargan (Brown, 2003). Sumado a esto y aunque se sabe que existen organismos de origen bacteriano o vegetal que pueden ser utilizados en el proceso de remediación de sitios contaminados también se sabe que muchos de estos organismos pueden producir compuestos intermedios en diversas rutas de oxidación que podrían exhibir mayores efectos tóxicos sobre la biota del sitio afectado que los contaminantes originales. Además, el uso de combustibles derivados del petróleo en los motores de combustión interna convencionales también genera compuestos resultantes de la oxidación parcial de estos y sus impurezas que contribuyen apreciablemente al aumento de la producción de gases de efecto invernadero como CO_2 , CH_4 , NO_x y SO_x (Escobar et al., 2008).

Por lo tanto, al buscar estrategias para abordar la necesidad de explotar fuentes de energía alternativas, no solo es necesario considerar factores técnicos o económicos, sino también generar estrategias para proporcionar valor agregado

a la operación, que apunta a aumentar la tasa de éxito del proceso para escalar hasta el nivel comercial sin sufrir presiones indebidas desde una perspectiva económica (Dufey, 2006).

Dentro del progreso general realizado en materia de investigación y desarrollo de tecnologías energéticas alternativas existe un vasto conjunto de tecnologías destinadas a aprovechar la luz solar, las corrientes de aire, la actividad geotérmica o la energía nuclear. Sin embargo, aunque existe una amplia gama de posibilidades, la mayoría de las tecnologías actualmente desarrolladas para la explotación de estas fuentes tienen muy bajos rendimientos de conversión y altos costos de infraestructura asociados con su explotación. Además, debe considerarse que este tipo de fuentes de energía alternativas a menudo se limitan a su aplicación para generar electricidad, pero no pueden cubrir fácilmente el creciente problema del suministro de combustible para el sector del transporte, donde la presencia de combustibles fósiles es más marcada (Melaina et al. al., 2013). Los intentos de incorporar las tecnologías antes mencionadas no solo requieren la implementación, por ejemplo, de un sistema de paneles solares, sino también la modificación del motor y su sistema mecánico para hacerlo compatible con esta nueva tecnología, y que surgiría la necesidad de reemplazar casi todos los vehículos existentes hoy en día por aquellos que podrían funcionar con el nuevo suministro de energía, lo que implica más problemas económicos y sociales (Ogden et al., 2004).

Cabe destacar que ninguna de las tecnologías presentadas en el párrafo anterior cumplen los criterios mencionados anteriormente de agregar beneficios adicionales fuera de la reducción de la contaminación derivada de su uso, especialmente considerando que otro tema preocupante es la tasa actual de generación de desechos y la contaminación ambiental causada por las diversas actividades humanas y el uso de energías renovables como eólica y solar no brindan respuestas directas a dichos problemas.

Los biocombustibles, que pueden definirse como todos los compuestos de naturaleza orgánica derivados de los seres vivos y su metabolismo que potencialmente pueden utilizarse como combustibles, lideran un grupo de fuentes de energía alternativas destinadas a dar solución a los problemas planteados anteriormente, ya que parecen más adecuados para abordar problemas específicos que otras tecnologías no pueden superar, como el uso de residuos orgánicos como materia prima (Maddipati et al., 2011). Además, los procesos de biocombustibles tienen un amplio conocimiento teórico que los respalda, ya que la tecnología de fermentación es casi tan antigua como la humanidad (Naik et al., 2010). Sin embargo, la investigación y el desarrollo actuales sobre tecnologías de biocombustibles no pueden alcanzar niveles de producción razonables para hacerlos atractivos desde el punto de vista económico debido tanto a los bajos rendimientos de conversión como a la disponibilidad de materiales de materias primas.

Biocombustibles

Los biocombustibles se pueden clasificar de acuerdo con el estado de agregación en el que son útiles como combustibles. Dentro de los biocombustibles gaseosos, el más representativo es el llamado biogás, que es una mezcla que consiste principalmente en metano, dióxido de carbono y otros elementos traza. Este tipo de combustible también se encuentra con relativa facilidad en el medio ambiente y puede provenir de fuentes naturales, como pantanos o lagos, o de fuentes antropogénicas como el ganado. La principal virtud de este tipo de compuestos es su alto valor calorífico, por lo que no se requieren grandes volúmenes de gas para lograr una producción aceptable de energía. Sin embargo, los problemas más importantes a superar antes de su uso como fuente de energía confiable son la gran cantidad de infraestructura necesaria para recolectar eficientemente tales compuestos y la dificultad y riesgo para su transporte y almacenamiento, ya que la presión y temperatura de los recipientes y conductos empleados para tales tareas necesitan un control estricto para evitar explosiones.

Los biocombustibles sólidos, compuestos principalmente de biomasa vegetal como madera, paja o carbón son abundantes en la naturaleza y el proceso de transformación que se requiere para transformarlos en combustibles útiles es menor o inexistente, sin embargo, su combustión incontrolada sí resulta en la generación de cenizas con altos contenidos de metales pesados o emisiones gaseosas que contienen azufre u óxidos de nitrógeno que no solo contribuyen a aumentar el efecto invernadero, sino que a su vez pueden formar ácidos fuertes en presencia de vapor de agua atmosférico y luego precipitarse como lluvia ácida (Vamvuca et al., 2003). Además, el proceso en el cual se pueden obtener, puede afectar aún más el equilibrio ecológico al requerir la destrucción de ecosistemas forestales a gran escala.

Finalmente, dentro de los biocombustibles líquidos, existe una amplia gama de compuestos pero, a diferencia de los biocombustibles sólidos o gaseosos, los líquidos no se encuentran fácilmente en el medio ambiente. En general, estos se obtienen principalmente a través de procesos de fermentación realizados por microorganismos de origen bacteriano o fúngico, o generados por algas con alto contenido de lípidos (Gomez et al., 2008). Dentro de este escenario no se puede anticipar que los biocombustibles líquidos compartirían la mayoría de los problemas de explotación, manejo o distribución de los sólidos y gaseosos, ya que la mayoría de los sistemas de combustión interna se basan en el uso de combustibles líquidos; sin embargo, estos compuestos aún sufren de bajos rendimientos de conversión y, en algunos casos, altos costos de recuperación de productos terminados, en contraste con los procesos de producción de combustibles fósiles clásicos, ya que tienden a tener solo un compuesto de interés recuperable o rentable, y eso afecta a la viabilidad económica de su producción (Pfromm et al., 2010).

Retos actuales en la producción de biocombustibles líquidos

Uno de los procesos actualmente establecidos a gran escala es la producción de etanol como aditivo o sustituto de la gasolina en motores de combustión interna,

que a pesar de ser una de las técnicas biotecnológicas más antiguas y contar con amplios conocimientos derivados de esa vasta experiencia, aún se requieren esfuerzos adicionales para un candidato sólido a largo plazo para reemplazar los combustibles fósiles (Lee et al., 2008). En primer lugar, se puede mencionar que el tipo de materia prima utilizada para la producción en Brasil y en los Estados Unidos, que son los mayores productores de etanol para uso como biocombustible, proviene de recursos originalmente destinados a materias primas animales y humanas, siendo azúcar caña y maíz respectivamente (Pimentel et al., 2007). Además, el etanol tiene limitaciones técnicas de uso, almacenamiento e integración en la tecnología actual del motor de combustión interna, como una mayor higroscopicidad con respecto a los combustibles a base de petróleo, por lo que no se puede transportar de manera eficiente en largos tramos sin acumular humedad adicional, por lo tanto reduciendo su rendimiento y contribuyendo a la oxidación interna de dichos tubos (Antony y Zverlov, 2007).

Con lo anterior, se han buscado diferentes biocombustibles en la naturaleza para tratar de disminuir los problemas mencionados anteriormente, y uno de los candidatos más convincentes es el butanol.

Butanol como biocombustible

El butanol es un alcohol de cadena corta que se ha obtenido desde hace mucho tiempo por medios biológicos y presenta la ventaja de tener un mayor contenido de energía por litro de combustible que el etanol y menos volatilidad y un índice de octano ligeramente menor que la gasolina, lo que puede mejorar su rendimiento el primero y reducir la cantidad de emisiones gaseosas de los motores frente a este último. También su mayor densidad conduce a una mayor cantidad de combustible que se inyecta en el motor, que teniendo en cuenta su poder calorífico similar frente a la gasolina, le ayuda a obtener un contenido de energía comparable por litro contra ella. Por lo tanto, se cree que el butanol sería un mejor diluyente de combustible o un sustituto de la gasolina regular actual que el etanol (Cuadro 1).

Cuadro 1. Comparación de diferentes propiedades fisicoquímicas entre gasolina regular, etanol y n-butanol (Modificado de Mužíková et al., 2014).

Combustible	Densidad (kg/m³)	Presión de Vapor (kPa)	Contenido Energético (MJ/L)	Octanaje de Investigación (RON)	Solubilidad en agua @ 20 °C (w/w)
Gasolina (US/UK)	740	45 - 90	32.6	95	0.01
n-butanol	810	2.3	29.2	94	20.1
Etanol	794	17	23.5	106	Miscible

Tradicionalmente, la metodología para obtener butanol por fermentación se basa en la degradación de diversos azúcares (particularmente glucosa o sacarosa) llevada a cabo por bacterias Gram-positivas del género *Clostridium*, a través de una vía metabólica llamada ABE (acetona-butanol-etanol) (Qureshi et al., 2008). Este proceso biológico se explotó originalmente para la producción de acetona como disolvente para la industria química y militar, teniendo tal importancia que la mayoría de los explosivos fabricados y utilizados durante la Segunda Guerra Mundial se basaron en esta tecnología. En aquel entonces, el butanol se consideraba un producto indeseable, sin embargo, en la antigua Unión Soviética comenzó su uso como combustible, en parte debido a las duras condiciones climáticas. A mediados de los años 60, el proceso de obtención de productos ABE a escala industrial permitió alcanzar volúmenes de reacción de hasta 300 m³. No obstante, el auge de la industria petroquímica en los años 70 proporcionó solventes y productos químicos a un costo muy bajo en comparación con el proceso biológico, por lo que las plantas de fermentación se desmantelaron (Zverlov et al., 2006).

Actualmente, debido a los motivos descritos en el párrafo inicial, ha habido un renovado interés en el estudio del sistema ABE para adaptar dicha tecnología a las demandas energéticas de esta época (Lee et al., 2008). Aun así, el sistema

biológico presenta restricciones nativas que han impedido su consolidación como una tecnología madura como bajos rendimientos de producción, efecto inhibitorio sobre el crecimiento del cultivo debido a la acumulación de solventes y mecanismos de regulación metabólica propios de bacilos Gram-positivos tales como el proceso de esporulación (Zheng et al., 2009).

Ruta metabólica de la fermentación ABE

La vía metabólica de ABE se compone de 19 reacciones principales (Figura 1), en las que el acetato, el butirato, el etanol, la acetona, el lactato y el hidrógeno son subproductos de la fermentación.

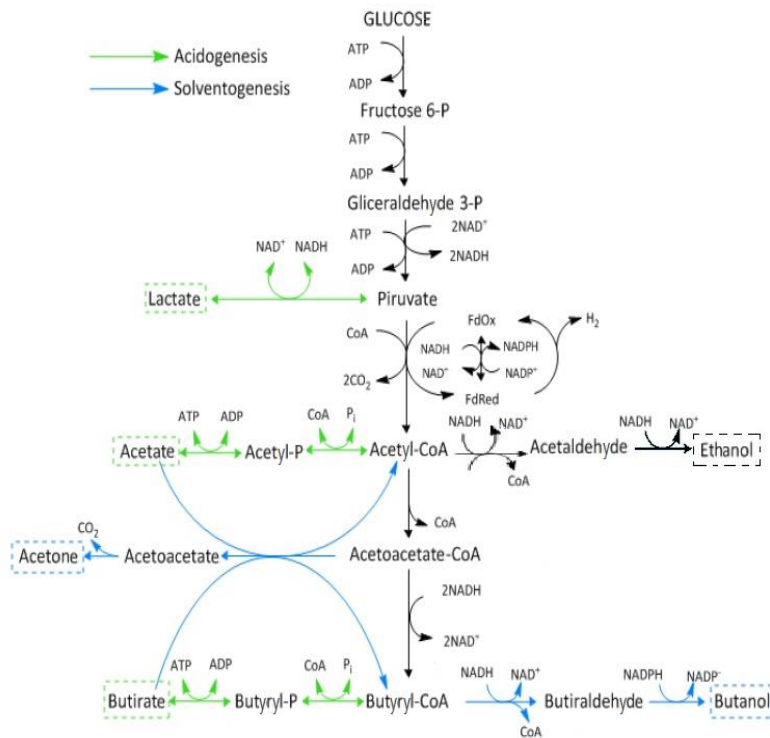


Figura 1. Diagrama de los 19 nodos principales que componen la vía metabólica ABE del género *Clostridium* (Modificado de Liu et al., 2013).

El metabolismo muestra dos fases distintas, una fase acidogénica, en la que el acetato y el butirato son los productos principales, y está relacionado con la fase de crecimiento exponencial del cultivo, y una fase solventogénica, en la que el butanol y la acetona son los productos principales, y más relacionado con la fase estacionaria. Se informa ampliamente que la mayoría de las bacterias

clostridiales producen etanol constitutivamente durante cualquiera de las dos etapas metabólicas exhibidas por el cultivo (Bahl et al., 1982).

Las hexosas se metabolizan en piruvato a través de la vía Embden-Meyerhof-Parnas, mientras que las pentosas se incorporan al metabolismo mediante la acción de la glucosa uridil transferasa UDP (Durán-Padilla et al., 2014). El piruvato es uno de los intermediarios clave del metabolismo de *Clostridium* y bajo ciertas condiciones las especies de *Clostridium* son capaces de transformar el piruvato en lactato a través de la piruvato deshidrogenasa. Sin embargo, la reacción principal es la conversión de piruvato en acetil CoA a través de piruvato ferredoxin oxidoreductasa (Uyeda y Rabinowitz, 1971), con la reducción concurrente de ferredoxina, por lo que la formación de lactato no se considera en la mayoría de los modelos metabólicos.

Las NAD(P)H-ferredoxin oxidoreductasas son enzimas clave para el transporte de electrones en el género *Clostridium* (Gheshlaghi et al., 2009). En cultivos productores de ácido, la ferredoxina reductasa muestra alta actividad, se proponen dos razones para tal fenómeno, es probable que este efecto provenga de la necesidad de regenerar el NAD consumido en la reacción de gliceraldehido-3-P-deshidrogenasa, o podría ser eso como los cultivos acidogénicos están relacionados con la fase de crecimiento exponencial y un flujo elevado a través de esta reacción, y por lo tanto, se requiere una alta actividad enzimática para la posterior producción de energía. El mecanismo regulador de la oxidoreductasas de ferredoxina parece estar relacionado con la concentración de NAD y NADH, que resulta ser un sistema regulador eficiente que evita la acumulación de NADH durante la fase acidógena y aumenta la actividad de NAD(P) reductasas durante la fase solventogénica, a su vez una mayor demanda de NAD(P)H para la producción de etanol y butanol en dicha etapa (Gheshlaghi et al., 2009).

La formación de hidrógeno es catalizada por la hidrogenasa, que usa ferredoxina reducida como donador de electrones (Gheshlaghi et al., 2009). Además de las

ferredoxin reductasas, la hidrogenasa muestra un comportamiento distintivo durante la acidogénesis y la solventogénesis, ya que la ferredoxina reducida compite con la NAD (P) - reductasas, lo que significa que también compite por reducir la potencia con butanol y etanol deshidrogenasas durante la solventogénesis.

La vía ABE tiene tres nodos importantes en Acetyl-CoA, Acetoacetyl-CoA y Butyryl-CoA. Acetil-CoA es un nodo rígido desde el cual el flujo de carbono se distribuye a cinco metabolitos diferentes. Dos ramas salen directamente de Acetyl-CoA que terminan en acetato y etanol, de Acetoacetyl-CoA se produce acetona y de Butyryl-CoA se producen butirato y butanol. El acetato y el butirato son reacciones productoras de energía en las que se produce 1 molécula de ATP por cada Acetilo / Butiril-CoA consumido. Ambos compuestos se vuelven a asimilar durante la solventogénesis, esta reacción se cataliza de dos maneras diferentes, el acetato y el butirato se pueden convertir en acetilo / butiril-CoA a través de las reacciones reversibles o mediante la acción de la CoA-transferasa (Millat et al. 2014), que consume una molécula de Acetoacetil-CoA y una de acetato / butirato para producir una molécula de acetoacetato y otra de acetilo / butiril-CoA. Este mecanismo establece una estrategia eficiente de producción de solventes, dado que sería energéticamente desfavorable volver a asimilar acetato y butirato a través de las reacciones reversibles, la disponibilidad de una reacción no consumidora de ATP le permite al organismo enfrentar el déficit de energía mientras sigue siendo capaz para producir etanol y butanol en la fase estacionaria o solventogénica.

Esta teoría está respaldada por los hallazgos de Desai et al. (1999) y Lehmann et al. (2012) que indica que el consumo de butirato durante la solventogénesis se cataliza a través de la reacción reversible, frente al consumo de acetato que está relacionado con la producción de acetoacetato. También Wang et al. (2013) y Desai et al. (1999) informan que el acetato se produce incluso después de que el organismo cambió a la solventogénesis, lo que sería una indicación de que la producción de acetato es necesaria para la generación de energía.

Existen varias hipótesis que intentan explicar los factores que controlan el cambio entre el metabolismo generador de ácido y el metabolismo generador de disolvente. Algunos autores (Hüsemann y Papoustakis, 1988; Terracciano y Kashket, 1986) sugieren que la concentración de ácido butírico no disociado es el factor de control. Sin embargo, Chen y Blaschek (1999) propusieron que el cambio es el resultado de señales extracelulares e intracelulares, como el pH del cultivo o las concentraciones intracelulares de acetil-P y butiril-P, respectivamente. Zhao y Tomas (2005) concluyeron que Butyryl-P y no Acetyl-P son las especies que controlan el metabolismo, mientras que Wietzke y Bahl (2012) sugieren que la energía y el equilibrio redox son las señales intracelulares clave para comenzar la producción de solventes.

Retos a superar en el proceso de fermentación ABE

Aunque, como se describió anteriormente, se puede suponer que el problema de la disponibilidad de sustrato para la producción de butanol como biocombustible podría garantizarse, debe mencionarse que, en general, la mayoría de los obstáculos que impiden el uso de cepas clostridiales en bioprocesos productores de ABE implican las limitaciones bioquímicas del crecimiento bacteriano por las concentraciones de sustrato y solventes en el caldo. Algunos autores (Lee et al., 2008; Qureshi et al., 2010) informan que las bacterias productoras de ABE no muestran crecimiento en soluciones que contienen más de 175 g L^{-1} de glucosa o de 14 a 21 g L^{-1} de solventes, ya que el exceso en la fuente de carbono interrumpe su incorporación por los microorganismos y los títulos elevados de solvente solubilizan parcialmente la pared de la célula y causa una despolarización de sus membranas, lo que impide la estabilización de dichas estructuras después o durante la división celular.

Los avances de investigación realizados hasta la fecha para resolver algunos de los problemas mencionados anteriormente tanto de la biología molecular como de las técnicas de ingeniería. En cuanto a la manipulación genética de las cepas

Hay informes de que la sobreexpresión de un factor de transcripción clave que regula la esporulación de la *Spo_a* denominada cultura junto con la regulación a la baja de la expresión de un sistema de hidrogenasas codificadas por el grupo de genes conocido como *hup_{CBA}* intensifica la producción de todo el disolventes en cultivo discontinuo y reduce el efecto inhibitorio causado por la acumulación de dichos compuestos en el medio (Alsaker et al., 2004). Además, hay informes de ingeniería metabólica para redirigir el flujo de carbono de las vías de formación de acetona a las que producen butanol, que se ha realizado empleando ARN antisentido que se dirige a la transcripción del gen *ctf_B* con éxito moderado (Tummala et al., 2003).

Estudios recientes de la enfoque de ingeniería han explorado la viabilidad de la aplicación de estrategias para las técnicas de cultivo convencionales en las que se propone para alimentar a un primer reactor directamente con residuos triturados agrícola e inocular con bacterias de *Clostridium thermocellum* para obtener azúcares fermentables de ella que podía alimentar a un segundo reactor inoculado con *Clostridium acetobutylicum* o *Clostridium beijerinckii* para obtener productos ABE (Qureshi et al., 2007). Dichos estudios han demostrado que el éxito de dicha tecnología puede mejorarse aún más implementando un cocultivo de una cepa productora de celulolíticos y una de butanol en el mismo reactor (Nakayama et al., 2011).

Incluso teniendo en cuenta todos los avances en el campo de la tecnología ABE mencionados previamente, estos no han podido lograr un uso óptimo de las capacidades de las cepas de *Clostridium* ya que generalmente se generan mediante estrategias basadas en conocimiento empírico y por lo tanto no pueden evaluarse eficientemente con técnicas de ingeniería, como análisis, diseño, optimización y control de procesos.

Intensificación de procesos como solución

Una de las posibles alternativas para atacar las problemáticas mencionadas es la denominada Intensificación de Procesos, la cual es un conjunto de metodologías encaminadas al mejoramiento de procesos existentes o al diseño de otros nuevos con respecto a productividad, eficiencia energética, reducción de costos y minimización del impacto ambiental utilizando herramientas de análisis, optimización y control (Lutze *et al.*, 2010; Nikačević *et al.*, 2012).

A pesar de que la concepción de Intensificación de Procesos existe desde la década de los 80, aún no hay un consenso acerca de la metodología más adecuada para alcanzar sus objetivos, sin embargo Lutze *et al.* (2010, 2013) han planteado un diagrama general de pasos a seguir para lograrlo, mismo que se ejemplifica en la Figura 2.

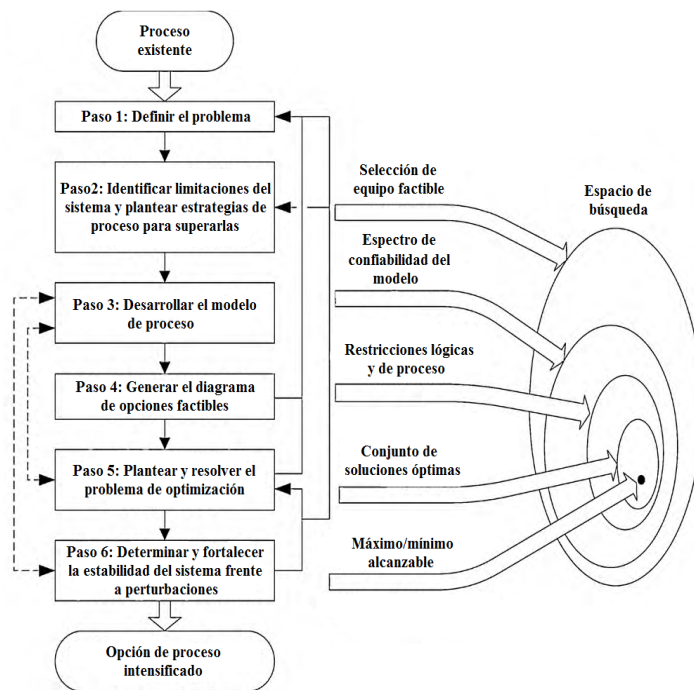


Figura 2. Diagrama de flujo propuesto por Lutze *et al.* (2010) para la Intensificación de Procesos.

Como se puede apreciar, el éxito de la implementación de estrategias de Intensificación de Procesos depende en gran medida de la adecuada construcción o selección de un modelo de planta que presente la mayor

capacidad posible para reproducir y predecir el comportamiento del sistema evaluado bajo una gran cantidad de condiciones operativas candidatas a darle solución al problema. Sin embargo, también es necesario conocer los límites o restricciones que presenta el fenómeno encargado de la transformación de la materia prima a los productos de interés y los requisitos que se necesitan cumplir para asegurar la viabilidad técnica y económica del proceso a desarrollar.

Modelado matemático del sistema de fermentación ABE

Las técnicas de simulación y modelado matemático se pueden utilizar como el primer paso en el diseño de nuevos procesos y biorreactores, a través del análisis del comportamiento del cultivo en diferentes condiciones y regímenes operativos, donde la literatura actual tiene una amplia variedad de estructuras propuestas para la descripción del proceso de fermentación ABE (Mayank, Ranjar y Moholkar, 2013; Millat y Winzer, 2017).

Cuadro 2. Modelos matemáticos informados previamente para la descripción del sistema de fermentación ABE.

Modelo	Contribución
Papoustakis et al., 1984	Primer modelo de fermentación utilizado para determinar los rendimientos del producto bajo el régimen de lotes a través de un balance de carbono
Votruba et al., 1986	Primer modelo dinámico para la fermentación por lotes en considerar un término no lineal para la inhibición del producto
Honda et al., 1987	Modelo dinámico hecho para simular el rendimiento de la productividad de butanol en lotes, lote alimentado y operación repetida de lote considerando una concentración de biomasa constante después de cada etapa de fermentación

Mulchandandi and Volesky, 1986	Modelo para la descripción de un sistema de fermentación ABE que opera en continuo con una unidad de retención celular y que utiliza un término discreto para describir la inhibición por butanol en función de los umbrales de concentración conocidos.
Shinto et al., 2007	Modelo metabólico compuesto por 19 ecuaciones cinéticas que representan cada una de las principales enzimas involucradas en la vía de fermentación ABE
Raganati et al., 2015	Modelo cinético para el sistema de fermentación ABE que opera en régimen discontinuo considerando diversas fuentes de carbono y su efecto en la tasa máxima de crecimiento del cultivo.
Eom et al., 2015	Modelo dinámico aplicado a la descripción de una unidad de recuperación de butanol ex-situ mediante adsorción acoplada a la fermentación ABE en régimen continuo usando un enfoque Monod-Ludeking-Piret y Langmuir extendido.
Thorn and King, 2016	Modelo dinámico del efecto de los cambios de pH aplicado a un cultivo continuo de <i>Clostridium acetobutylicum</i> basado en la representación de las etapas acidogénica y solventogénica por inclusiones diferenciales.

Sin embargo, incluso considerando que el modelado matemático del sistema de fermentación ABE tiene al menos 40 años de desarrollo, no hay consenso sobre la estructura más adecuada para usar en el ámbito del diseño, control y optimización de procesos, ya que la gran mayoría de las estructuras modelo informadas, solo son válidas bajo condiciones y regímenes de procesos específicos. Recientemente, el llamado enfoque de modelado cinético fenomenológico no estructurado, buscado encontrar el punto medio entre la complejidad de los modelos de nivel metabólico o genómico y la facilidad de uso e interpretación de los modelos cinéticos no estructurados tradicionales, este

enfoque se ha aplicado con éxito al sistema ABE y es una perspectiva sólida para evaluar el rendimiento de este sistema bajo regímenes más complejos como los que implican optimización media, clústeres de reactor de etapas múltiples o sistemas integrales que implican inmovilización celular y estrategias de recuperación *in situ* (Velázquez-Sánchez et al., 2014).

Optimización de procesos aplicado al sistema ABE

La optimización del proceso de fermentación ABE ha sido durante mucho tiempo un objetivo de la industria de bioprocesos. Los procesos experimentales actualmente desarrollados incluyen el reciclaje de células y los reactores de inmovilización celular con el fin de aumentar la densidad celular, la productividad y el uso de fermentaciones extractivas *in situ* para minimizar la inhibición del producto. Sin embargo, a pesar de los esfuerzos mencionados anteriormente, los mejores resultados obtenidos para las fermentaciones ABE hasta la fecha son todavía menos del dos por ciento en concentración de butanol, $4.46 \text{ g L}^{-1} \text{ h}^{-1}$ de productividad y un rendimiento de solvente de menos de 25 % a partir de glucosa y por lo tanto, ha demostrado que no son lo suficientemente eficientes en relación con el tiempo y el esfuerzo necesarios para su implementación (Khöler et al., 2015).

Los esfuerzos de investigación realizados en el último lustro para lograr mayores concentraciones de producto y rendimientos han tomado diferentes enfoques para superar las barreras biológicas y fisicoquímicas para lograr una verdadera viabilidad económica. Por ejemplo, el trabajo realizado por Eom et al. (2015) se enfoca en el establecimiento y modelado de un sistema de fermentación ABE acoplado con una unidad de recuperación de butanol ex-situ con resultados prometedores, sin embargo utilizan una representación cinética bastante inexacta de la etapa de fermentación debido a la utilización de un tipo Ludeking-Piret estructura para las tres variables de estado consideradas (sustrato, biomasa y butanol), que limitan sus hallazgos solo al régimen operacional evaluado experimentalmente.

Un estudio más reciente realizado por Gallardo et al. (2016) hace uso del análisis dinámico de un modelo de escala del genoma del sistema de fermentación ABE para determinar la respuesta del sistema a la absorción del suministro externo de electrones en el medio de cultivo debido a la adición de ácido butírico, e incluso si dicho trabajo propone directrices operativas para encontrar la alimentación óptima de ácido butírico para lograr la máxima producción de butanol; su trabajo asegura que el punto óptimo obtenido no corresponde a la tasa máxima de crecimiento del cultivo, y también dichos resultados son solo válidos para cultivos bajo régimen de lote.

Finalmente, Kim et al. (2017) aplicaron un enfoque de optimización basado en modelos para maximizar las ganancias económicas de una fermentación ABE continua junto con un proceso de adsorción aguas abajo, e incluso si sus resultados muestran que fue posible alcanzar una productividad de butanol superior a $3 \text{ g L}^{-1} \text{ h}^{-1}$ el hecho de que una de sus restricciones operativas era mantener la concentración de butanol por debajo de 8 g L^{-1} , y también que su representación cinética elegida de la etapa de fermentación se basa nuevamente en una representación de Monod o Ludeking-Piret, junto con el hecho de la mayoría de las especies de *Clostridium* no son genéticamente estables para ser cultivadas en régimen continuo durante más de 500 h hace que esos resultados no sean confiables.

Como la mayoría de los mencionados anteriormente, típicamente en la fermentación ABE, las condiciones que conducen a una alta concentración de butanol hacen que el cultivo sea incapaz de mantener un perfil de alta productividad. En este sentido, las técnicas de optimización multiobjetivo tienen la ventaja de proporcionar el conjunto de soluciones no dominadas para dicho comportamiento conflictivo en forma de frentes de Pareto, que pueden usarse para determinar las mejores condiciones para satisfacer ambos objetivos (Houska et al., 2011).

Optimización multiobjetivo

Como lo expresan Villaverde et al. (2016), la productividad es la medida industrial para la viabilidad económica de un bioproceso. Se define como la velocidad a la que se producen los metabolitos objetivo y, por lo tanto, es una medida del rendimiento del cultivo para producir el producto deseado. Sin embargo, en el caso del sistema de fermentación ABE donde los productos deseados deben recuperarse del reactor usando procesos de alta energía como la destilación o metodologías altamente sensibles como la pervaporación o las operaciones de membrana, la concentración de tales metabolitos debe maximizarse para reducir tanto como sea posible, los costos indirectos del sistema.

Recientemente, en el marco del control y diseño óptimo de bioprocesos, se presta cada vez más atención a la optimización de múltiples objetivos (MOO) para la solución de los problemas descritos en el último párrafo. Contrariamente a la optimización de objetivo único (SOO), MOO puede dar cuenta de criterios tan diferentes y, por lo tanto, proporciona al diseñador información sobre las ventajas y desventajas involucradas entre los diferentes objetivos que se desean optimizar (Hreiz et al., 2015).

Matemáticamente hablando, un problema de optimización dinámica (DOP) se puede describir como:

$$\min_{x(\cdot), u(\cdot), p} J = \int_{\xi_0}^{\xi_f} \mathcal{L}(x(\xi), u(\xi), p) d\xi + \mathcal{M}(x(\xi_f), p) \quad (1a)$$

$$\text{tal que: } 0 = f(\dot{x}(\xi), x(\xi), u(\xi), p, \xi); \quad \xi \in [\xi_0; \xi_f] \quad (1b)$$

$$0 = b_i(x(0), p) \quad (1c)$$

$$0 \geq c_p(x(\xi), u(\xi), p, \xi) \quad (1d)$$

$$0 \geq c_t(x(\xi), u(\xi), p, \xi_f) \quad (1e)$$

donde \mathbf{x} representa las variables de estado, \mathbf{u} las variables de control, y \mathbf{p} los parámetros fijos. La dinámica del proceso se define mediante un conjunto de

ecuaciones dinámicas f sobre el intervalo de la variable independiente $\xi \in [\xi_0; \xi_f]$. Debe observarse que aunque la solución de control óptima se obtiene durante el intervalo de la variable independiente, el valor final (ξ_f) también puede ser una variable de optimización, por ejemplo, el tiempo final en la operación por lotes. Las condiciones iniciales del sistema están contenidas en las restricciones de igualdad de Eq. 1c, mientras que las Eqs. 1d-1e representan las restricciones de ruta y terminales que actúan en el sistema. El objetivo funcional puede consistir tanto en el término de Lagrange \mathcal{L} como en el de Mayer \mathcal{M} . Como en este caso de estudio, f es un sistema de ecuaciones diferenciales ordinarias (ODE) y todas las funciones son dos veces diferenciables.

La formulación general del problema óptimo multiobjetivo (MOOP) es similar al DOP, con la excepción de que la función objetivo ahora se convierte en una secuencia de funciones. Deje que el vector \mathbf{z} contenga todas las variables de optimización, es decir, $\mathbf{z} = [\mathbf{x}^T, \mathbf{u}^T, \mathbf{p}^T]$ y el problema de optimización se convierte entonces en:

$$\min_{\mathbf{z}} [J_1(\cdot), J_2(\cdot), \dots, J_m(\cdot)] \quad (2a)$$

$$\text{tal que } \mathbf{z} \in \mathcal{S} \quad (2b)$$

donde \mathcal{S} define el conjunto factible que satisface las restricciones de las Eqs. 1b-1e. Para los MOOP, no se puede obtener una solución única que optimice todos los objetivos (posiblemente contradictorios). En contraste, el concepto de "dominio de Pareto" se usa para comparar soluciones: una solución factible domina a otra solo si conduce a un rendimiento superior o igual con respecto a todos los criterios de evaluación. Se dice que una solución es "óptima de Pareto" si no está dominada por ninguna otra solución factible, y tal enfoque conduce a la generación de un conjunto de soluciones óptimas conocido como el frente de Pareto (PF) (Nimmegeers et al., 2016).

Estabilidad en sistemas de fermentación

Es bien sabido que los reactores biológicos normalmente se rigen por un comportamiento altamente no lineal que se produce tanto en la escala del reactor macroscópico como en la escala celular microscópica, la simulación dinámica es una herramienta importante y útil para la validación del modelo, pero se pueden identificar algunos inconvenientes; para los sistemas con comportamiento transitorio lento, el análisis dinámico es ineficiente y potencialmente no concluyente. De lo anterior, es importante encontrar nuevas formas de analizar tales sistemas que podrían proporcionar una descripción más detallada de su desempeño bajo una amplia gama de condiciones operacionales.

El análisis de bifurcación es una herramienta poderosa para evaluar modelos dependientes del tiempo aplicados a biorreactores continuos. El objetivo de la teoría de la bifurcación es caracterizar los cambios en el comportamiento dinámico cualitativo de un sistema no lineal ya que los parámetros clave son variados, generando una imagen del comportamiento del modelo en forma de diagrama de bifurcación, que puede utilizarse para determinar si el modelo admite el comportamiento estable y dinámico observado experimentalmente (Elnashaie, et al., 2007). Cuando se aplica este análisis considerando parámetros clave como los de bifurcación, es posible predecir el conjunto de condiciones operativas que podrían llevar al sistema a un conjunto de estados estables de alta producción y discernir cuál de ellos es estable o no.

Además, un control de biorreactor continuo es generalmente para evitar el lavado que podría provocar que la reacción se detenga para que pueda mantener una tasa de producción deseable. Esto se puede hacer cerrando un ciclo de retroalimentación y controlando la masa celular o las concentraciones de sustrato (Zhao y Skogestad, 1997).

En este sentido, la estrategia de control de la aplicación tempranamente exitosa en el control del proceso está en la evolución del controlador PID con el método

de ajuste tradicional de Ziegler-Nichols (Ziegler y Nichols, 1942). Hasta ahora, un alto porcentaje de los controladores implementados en las industrias de proceso son de tipo PID (Nikačević et al., 2012). Sin embargo, dado que el alto comportamiento no lineal del bioproceso y el mecanismo adaptativo de los microorganismos, es necesario diseñar nuevos algoritmos de control para mejorar el rendimiento del biorreactor y garantizar un régimen operativo de seguridad (Spear, 2005; Munasinghe y Nakamura, 2007; Xu y Yu, 2010). Recientemente, se han propuesto funciones alternativas acotadas para fines de estimación y control con un rendimiento satisfactorio (López-Pérez et al., 2015 y Aguilar-López et al., 2016).

Planteamiento teórico para la intensificación del sistema ABE

En el caso del sistema de fermentación ABE por *Clostridium acetobutylicum* Khöler et al. (2015) hace un análisis del desempeño de los sistemas de producción propuestos desde 1960 hasta la fecha y con base en ello propone un conjunto de restricciones mínimas que se deben de cumplir para considerar que un proceso es viable. Entre esas restricciones menciona que el mínimo de concentración de butanol a la salida o término del proceso no debe ser menor a 8 g/L, ya que de lo contrario no podría competir con el proceso tradicional por lote, además que en el caso ideal se debe superar la barrera de los 40 g/L para garantizar un balance positivo de energía si se realiza la recuperación del producto vía destilación, sin embargo Jones y Wood (1986) estimaron que el costo de separación de los solventes en una fermentación ABE reduce de manera lineal en el intervalo de 12 a 19 g/L de estos, por lo que cualquier incremento en la capacidad de producción del sistema se verá reflejado de manera directa en una reducción en el costo de los procesos *downstream*. A su vez, Jiang et al. (2015) reportan que actualmente se tiene conocimiento que existe una planta industrial de producción de ABE por fermentación que presenta las características mencionadas en la Figura 3; considerando que uno de los mayores costos en el proceso es la purificación del producto y por lo mencionado

anteriormente en este párrafo se hace necesario explorar nuevas alternativas de proceso que permitan abatir dicho inconveniente.

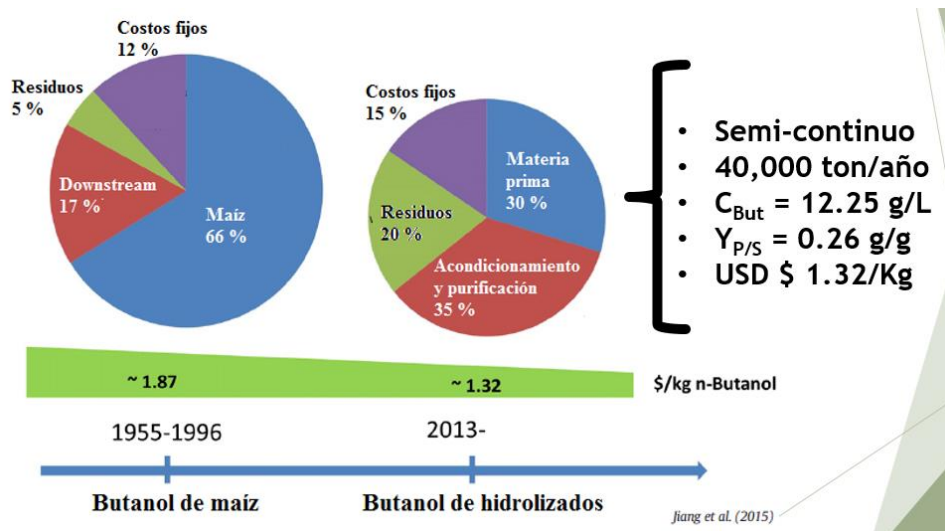


Figura 3. Caracterización del proceso industrial de producción de ABEs por vía fermentativa reportado por Jiang et al., 2015.

Con lo anterior entonces se propone el uso de la metodología de intensificación de procesos utilizando como objeto de estudio un sistema de fermentación ABE por *Clostridium acetobutylicum* para intentar diseñar un proceso capaz de cumplir con las restricciones mínimas antes mencionadas, con lo cual se pueda garantizar su competitividad económica con respecto al uso de combustibles fósiles.

Justificación.

Existe una amplia cantidad de trabajos reportados en la literatura encaminados a aumentar el desempeño del sistema de fermentación ABE por *Clostridium acetobutylicum*. Sin embargo, no hay todavía un estudio definitivo que pueda tomarse como base para el diseño de un proceso que pueda considerarse óptimo dentro del marco de restricciones establecidas por algunos autores para garantizar su viabilidad. Por tanto, la implementación de la metodología de Intensificación de Procesos sobre el sistema ABE puede ser una alternativa para el diseño de procesos de producción que cumplan con dichos requisitos.

Hipótesis.

La implementación de la Intensificación de Procesos sobre un sistema de fermentación ABE por *Clostridium acetobutylicum* ATCC 824 permitirá encontrar una estrategia de operación que supere el desempeño del sistema industrial reportado por Jiang et al. (2015) tanto en concentración como en productividad de butanol.

Objetivos

General:

Aplicar los fundamentos de Intensificación de Procesos para proponer un sistema de producción que permita aumentar la concentración y productividad de butanol por *Clostridium acetobutylicum* ATCC 824 con respecto al sistema de producción industrial reportado por Jiang *et al.* (2015).

Particulares:

- Construir y validar un modelo matemático para representar la dinámica del sistema de producción de butanol por *Clostridium acetobutylicum* ATCC 824.
- Seleccionar por medio de simulaciones el régimen de operación (lote, fed-batch con alimentación lineal o continuo con una o múltiples etapas) más factible para incrementar la concentración y productividad de butanol del sistema estudiado contra el proceso industrial reportado por Jiang *et al.* (2015).
- Caracterizar el comportamiento dinámico del sistema de producción de butanol por *Clostridium acetobutylicum* ATCC 824 bajo el régimen de operación seleccionado.
- Plantear y resolver el problema de optimización No Lineal que permita encontrar las condiciones de operación necesarias para maximizar la productividad de butanol del sistema seleccionado.
- Diseñar e implementar una retroalimentación en forma de ley de control para asegurar la estabilidad del sistema estudiado bajo condiciones de operación óptimas.

Metodología

La Figura 4 muestra de manera general la metodología a seguir durante el desarrollo del trabajo para el cumplimiento de los objetivos particulares del mismo.

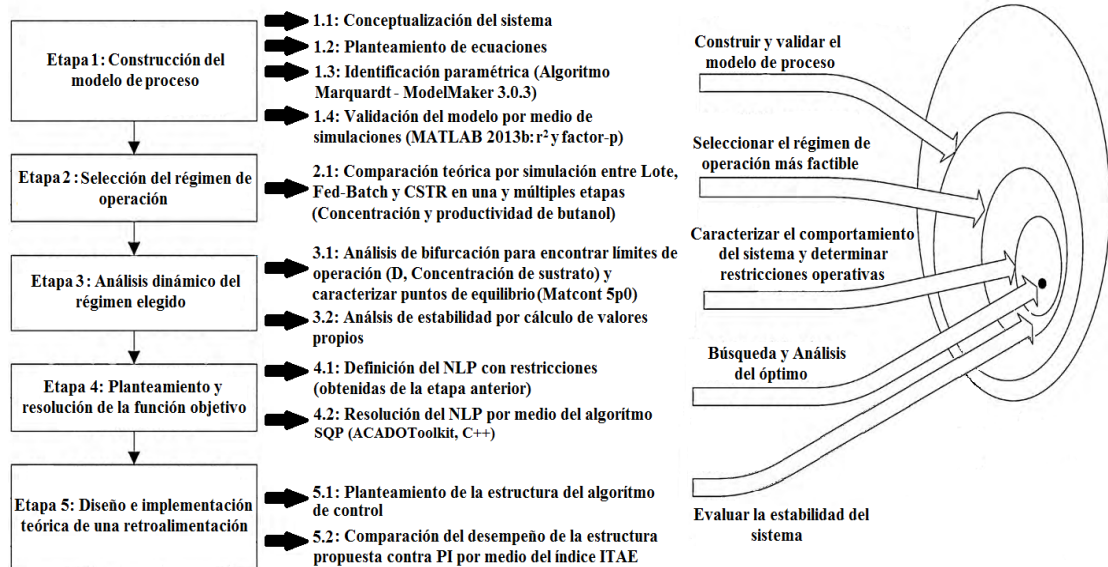


Figura 4. Diagrama que muestra las 5 Etapas en las cuales se divide el desarrollo del presente trabajo.

Construcción del modelo de proceso

El análisis de la vía metabólica simplificada de *Clostridium acetobutylicum* reportado por Liu et al. (2013), que se muestra en la Figura 1, se utilizó para proponer las estructuras matemáticas que representan las velocidades de reacción del sistema ABE. Describe que el metabolismo de la oxidación de glucosa se lleva a cabo en dos fases secuenciales, una fase llamada acidogénesis, llevada a cabo por células vegetativas, que incluye desde la ruta de glucólisis hasta la formación de Acetil CoA y su posterior oxidación a ácidos orgánicos como butírico, láctico y acético; y otra vía metabólica llamada solventogénesis, realizada en conjunto con el metabolismo que desencadena el proceso de esporulación, que comienza con la reincorporación de los ácidos orgánicos mencionados anteriormente a la célula y culmina con su

transformación en acetona y butanol. En el caso del etanol, se informa en la literatura que su producción es constitutiva, independientemente del estado metabólico del cultivo (Jones y Wood, 1986). También es importante señalar que la cepa *Clostridium acetobutylicum* ATCC 824 no ha sido modificada genéticamente para inhibir su proceso de esporulación, por lo que este efecto también se consideró dentro del desarrollo del modelo.

Identificación paramétrica y validación de los modelos

En un intento de comparar las capacidades descriptivas y predictivas de las diferentes estrategias de modelado aplicadas al sistema de fermentación ABE, los datos experimentales reportados por Yen y Li (2011), que consideran un reactor tanque agitado de 1 L de volumen nominal trabajando con 600 mL de medio P2 con glucosa como principal fuente de carbono e inoculada con células de *Clostridium acetobutylicum* ATCC 824, se consideró adecuada para alcanzar dicho objetivo.

En el trabajo mencionado anteriormente, el reactor se hizo funcionar bajo cuatro regímenes de operación diferentes: discontinuo y continuo con células libres, y discontinuo y continuo con células inmovilizadas respectivamente, donde el medio de inmovilización era ladrillo en polvo con un diámetro de partícula entre 0,15 y 2,4 mm.

La identificación paramétrica de los modelos propuestos se realizó mediante el algoritmo Levenberg-Marquardt en el software ModelMaker® 3.0.3.

El método de Levenberg-Marquardt es una expansión del método Gauss-Newton y también se utiliza con mínimos cuadrados no lineales. Utiliza el mismo proceso de paso iterativo, sin embargo, tiene una forma ligeramente diferente de la ecuación normal como se muestra en la Eq. 3:

$$(J^T J + \lambda I)s = -J^T \cdot r \tag{3}$$

El término λ se conoce como el factor de amortiguación. Cuando λ es pequeño, el método sigue el método de Gauss-Newton, sin embargo, cuando λ es grande, tiende hacia la dirección de descenso más pronunciada. El factor de amortiguación se cambia de forma adaptativa sobre iteraciones del método. Esto permite que el método de descenso más pronunciado tenga prioridad cuando está lejos del mínimo y el más rápido convergencia del método de Gauss-Newton para tomar el control cuando se está cerca del mínimo.

Las ventajas de este método de identificación paramétrica son que es un método robusto, que requiere pocas evaluaciones de las funciones a optimizar aun iniciando con aproximaciones muy lejanas al mínimo y que tiene la capacidad de realizar aproximaciones adaptativas entre algoritmos dependiendo de la cercanía a los valores óptimos del vector de parámetros libres (Dkhichi et al., 2014).

Todas las simulaciones numéricas se realizaron en una PC equipada con un procesador Intel® Xeon © E5460 y 8 GB de RAM en el software MATLAB® 2016a, utilizando el comando ode15s de la biblioteca ODESolver para resolver los diferentes conjuntos de ecuaciones diferenciales.

El rendimiento teórico de los modelos probados se evaluó aplicando un enfoque de coeficiente de determinación clásico a los tres modelos en comparación con los datos experimentales obtenidos por Yen y Li (2011) y Qureshi et al. (2007), analizando no solo el rendimiento global, sino también calculando la bondad del ajuste para cada una de las variables informadas. Además, para validar aún más las estructuras propuestas, se realizó un análisis complementario considerando el índice Π definido en la ecuación 4. El índice Π indica que el modelo tiene un rendimiento deficiente si su valor es negativo, si es exactamente 0 sugiere que el modelo puede reproducir al menos la media de la variable estudiada, si su valor es mayor que 0 pero inferior a 0.5, el rendimiento se considera aceptable y si el valor es mayor que 0.5, entonces el rendimiento del modelo se considera como adecuado.

$$\Pi = \mathbf{1} - \frac{\sum_{i=1}^N [|Y - Y^*|]}{\sum_{i=1}^N [|Y^* - \bar{Y}|]} \in (-\infty, \mathbf{1}] \quad (4)$$

Finalmente, para inferir las capacidades predictivas de las estructuras elegidas, las concentraciones experimentales y simuladas de butanol en estado de equilibrio en cultivo discontinuo y estado estacionario en régimen continuo se evaluaron usando un ensayo ANOVA para determinar las diferencias estadísticas puntuales sobre cada condición operacional particular probada y también calculando la bondad de ajuste para los modelos frente al comportamiento general del sistema de fermentación bajo la manipulación de la concentración de sustrato del reactor inicial (S_{g0}) en régimen de lote y la tasa de dilución (D) y la concentración de sustrato de alimentación (S_{gin}) bajo régimen continuo respectivamente.

Selección del régimen de operación

Se realizaron simulaciones numéricas en el software MATLAB® 2016a, utilizando el comando ode15s de la biblioteca ODESolver para resolver el conjunto de ecuaciones diferenciales del modelo cinético con mejor desempeño obtenido de la actividad anterior considerando los siguiente regímenes operacionales: lote con alimentación de ácido orgánico, CSTR de etapa única, CSTR de tres etapas con y sin recirculación de biomasa y lote alimentado con velocidad de alimentación constante. El rendimiento teórico del sistema se evaluó considerando el título de butanol y la productividad en el estado de equilibrio en el cultivo por lotes y el estado estacionario en todos los CSTR.

Además, para caracterizar el comportamiento de estado estable de los sistemas que operan bajo régimen continuo, se elaboraron diagramas de bifurcación considerando la tasa de dilución ($D = F/V$), la concentración de glucosa en la corriente de alimentación (S_{ga}) y la concentración de ácido butírico en la

alimentación (S_{b_a}) como parámetros de bifurcación utilizando el programa MATCONT versión 5p0, que se ejecuta en el marco de MATLAB® 2016a.

Optimización del desempeño del sistema de fermentación ABE

Para obtener las condiciones de cultivo que maximizaran la concentración y productividad de butanol en los regímenes de operación evaluados se recurrió a la aplicación de una estrategia de optimización multiobjetivo considerando el algoritmo de Descenso más Pronunciado para el régimen por lote utilizando código propio en MATLAB® 2016a considerando la concentración inicial de glucosa (S_{g_0}) y la de butirato (S_{b_0}) como parámetros a optimizar; y el de Programación Cuadrática Secuencial haciendo uso del programa ACADOToolkit versión 1.2 (Houska et al., 2014), mismo que se ejecuta dentro del marco de Visual® C++ 2013, con el cual se elaboraron los frentes de Pareto correspondientes a los regímenes semicontinuo y continuo considerando al flujo de alimentación (F), concentración de glucosa en la corriente de alimentación (S_{g_a}) y concentración de butirato en la corriente de alimentación (S_{b_a}) como variables de proceso a determinar.

Diseño e implementación de una retroalimentación

Se diseña un nuevo controlador no lineal con una clase de realimentación de salida sigmoidea limitada para proporcionar estabilización a una clase de biorreactor continuo para la producción de butanol. El controlador tiene como objetivo regular la concentración de butanol dentro del reactor al punto de referencia correspondiente y así llevar la productividad de butanol a valores más altos. La validación de los resultados se realizó comparando la productividad teórica máxima general del reactor a lazo abierto, frente al efecto de la ley de control propuesta en la operación a lazo cerrado.

Resultados y Discusión.

Construcción de los modelos cinéticos

Para la construcción del modelo tradicional se consideró la relación clásica de Ludeking-Piret para la generación del producto y la tasa de crecimiento de Monod-Levenspiel para la descripción de la dinámica de población bacteriana dentro de la fermentación para tener en cuenta el efecto inhibitor de la acumulación de sustrato y producto en el recipiente de reacción. Esta estructura se usó en los tres modelos construidos para evaluar el impacto de la complejidad y estructura del modelo con respecto a los parámetros cinéticos del núcleo como μ_{max} y la concentración inhibitora de glucosa y butanol, ya que se espera que dichos valores permanezcan relativamente cercanos en valor uno del otro, independientemente del modelo empleado para representar los datos experimentales.

$$\mu_X = \eta_1 \left(\mu_{max} * \left(\frac{Sg}{kSg+Sg} \right) \left(1 - \left(\frac{But}{kBut} \right) \right) \left(1 - \left(\frac{Sg}{ksi} \right) \right) \right) \quad (5)$$

$$\frac{dSg}{dt} = D(Sg_{in} - Sg) - \left(\frac{\mu_X \cdot X}{Y_{X/Sg}} \right) \quad (6)$$

$$\frac{dX}{dt} = -\psi DX + (\mu_X - k_{Spo})X \quad (7)$$

$$\frac{dBut}{dt} = -D(But) + \alpha_{But} \mu_X X + \beta_{But} X \quad (8)$$

$$\frac{dSb}{dt} = D(Sb_{in} - Sb) + \alpha_{Sb} \mu_X X + \beta_{Sb} X \quad (9)$$

$$\frac{dAct}{dt} = -D(Act) + \alpha_{Act} \mu_X X + \beta_{Act} X \quad (10)$$

$$\frac{dAce}{dt} = -D(Ace) + \alpha_{Ace} \mu_X X + \beta_{Ace} X \quad (11)$$

$$\frac{dEt}{dt} = -D(Et) + \alpha_{Et} \mu_X X + \beta_{Et} X \quad (12)$$

El modelo cinético está compuesto por la tasa de crecimiento (Ec. 5) y un conjunto de siete ecuaciones diferenciales (Ecs. 6-12) que describen los balances de masa correspondientes para Glucosa (**Sg**), Biomasa (**X**), Butanol (**But**), Butirato (**Sb**), Acetato (**Act**), Acetona (**Ace**) y Etanol (**Et**) concentraciones respectivamente. **D** representa la velocidad de dilución del reactor y **Sg_{in}** y **Sb_{in}** son las concentraciones de glucosa y butirato dentro de la solución de alimentación.

Para la construcción del modelo cinético estructurado se tomó en consideración el enfoque de Shinto et al. (2011) al representar cada uno de los 19 pasos enzimáticos de la ruta metabólica (Fig. 1) con una estructura de Michaelis-Menten. Para este escenario también se consideró que ambas reacciones en todos los pasos reversibles fueron hechas por enzimas individuales en lugar de por una única función de doble función. El modelo quedó compuesto por 15 ecuaciones diferenciales ordinarias (ODE) que describen el comportamiento de los metabolitos tanto extracelulares (Ecs. 13-20) como intracelulares (Ecs. 21-27) producidos en la fermentación ABE:

$$\frac{dSg}{dt} = D(Sg_{in} - Sg) - \left(v_{max1} \left(\frac{Sg}{Km1+Sg} \right) \right) X - \left(\frac{\mu_X}{Y_{XS}} \right) X \quad (13)$$

$$\frac{dX}{dt} = -DX + (\mu_X - k_{spo})X \quad (14)$$

$$\frac{dBut}{dt} = (D(-But)) + \left(v_{max19} \left(\frac{BCoA}{Km19+BCoA} \right) \right) X \quad (15)$$

$$\frac{dSb}{dt} = (D(Sb_{in} - Sb)) + \left(v_{max18} \left(\frac{BCoA}{Km18+BCoA} \right) X \right) - \left(v_{max17} \left(\frac{Sb}{Km17+Sb} \right) \left(\frac{1}{1 + \left(\frac{But}{kBut} \right)} \right) \right) X \quad (16)$$

$$\frac{dAct}{dt} = (D(-Act)) + \left(v_{max9} \left(\frac{ACoA}{Km9+ACoA} \right) \right) X - \left(v_{max7} \left(\frac{Act}{Km7+Act} \right) \right) X \quad (17)$$

$$\frac{dAce}{dt} (D(-Ace)) + \left(v_{max16} \left(\frac{AAtO}{Km16+AAtO} \right) \right) X \quad (18)$$

$$\frac{dEt}{dt} = (D(-Et)) + \left(v_{max11} \left(\frac{ACoA}{Km11+ACoA} \right) \right) X \quad (19)$$

$$\frac{dLac}{dt} = (D(-Lac)) + \left(v_{max5} \left(\frac{Pir}{Km5+Pir} \right) \right) X - \left(v_{max4} \left(\frac{Lac}{Km4+Lac} \right) \right) X \quad (20)$$

$$\frac{dF6P}{dt} = (D(-F6P)) + \left(v_{max1} \left(\frac{Sg}{Km1+Sg} \right) \right) X - \left(v_{max2} \left(\frac{F6P}{Km2+F6P} \right) \right) X \quad (21)$$

$$\frac{dG3P}{dt} = (D(-G3P)) + \left(v_{max2} \left(\frac{F6P}{Km2+F6P} \right) \right) X - \left(v_{max3} \left(\frac{G3P}{Km3+G3P} \right) \right) X \quad (22)$$

$$\begin{aligned} \frac{dPir}{dt} = & (D(-Pir)) + \left(v_{max3} \left(\frac{G3P}{Km3+G3P} \right) \right) X + \left(v_{max4} \left(\frac{Lacc}{Km4+Lacc} \right) \right) X - \\ & \left(v_{max5} \left(\frac{Pir}{Km5+Pir} \right) \right) X - \left(v_{max6} \left(\frac{Pir}{Km6+Pir} \right) \right) X \end{aligned} \quad (23)$$

$$\begin{aligned} \frac{dACoA}{dt} = & (D(-ACoA)) + \left(v_{max6} X \left(\frac{Pir}{Km6+Pir} \right) \right) + \left(v_{max7} X \left(\frac{Act}{Km7+Act} \right) \right) - \\ & \left(v_{max9} X \left(\frac{ACoA}{Km9+ACoA} \right) \right) - \left(v_{max10} \left(\frac{ACoA}{Km10+ACoA} \right) \right) X - \left(v_{max11} \left(\frac{ACoA}{Km11+ACoA} \right) \right) X \end{aligned} \quad (24)$$

$$\begin{aligned} \frac{dAACoA}{dt} = & (D(-AACoA)) + \left(v_{max10} \left(\frac{ACoA}{Km10+ACoA} \right) \right) X - \left(v_{max8} \left(\frac{AACoA}{Km8+AACoA} \right) \right) X - \\ & \left(v_{max14} \left(\frac{AACoA}{Km14+AACoA} \right) \right) X \end{aligned} \quad (25)$$

$$\frac{dAAtO}{dt} = (D(-AAtO)) + \left(v_{max8} \left(\frac{AACoA}{Km8+AACoA} \right) \right) X - \left(v_{max16} \left(\frac{AAtO}{Km16+AAtO} \right) \right) X \quad (26)$$

$$\begin{aligned} \frac{dBCoA}{dt} = & (D(-BCoA)) + \left(v_{max14} \left(\frac{AACoA}{Km14+AACoA} \right) \right) X + \left(v_{max17} \left(\frac{Sb}{Km17+Sb} \right) \right) X - \\ & \left(v_{max19} \left(\frac{BCoA}{Km19+BCoA} \right) \right) X - \left(v_{max18} \left(\frac{BCoA}{Km18+BCoA} \right) \right) X \end{aligned} \quad (27)$$

Para el modelo cinético no estructurado fenomenológico se consideró una representación de las velocidades de reacción individuales para cada una de las moléculas producidas siguiendo un enfoque de Michaelis-Menten (Ecs. 28-32) y un enfoque de balance de masa clásico para cada una de las variables modeladas (Ecs. 33-39). Debe observarse que a medida que la vía ABE se segmenta en las etapas de acidogénesis y solventogénesis, las ecuaciones de butirato y acetato tienen un término de generación y uno de consumo para reflejar dicho fenómeno.

$$v_{But} = \eta_2 \left(v_{maxBut} \left(\frac{Sb}{kSb+Sb} \right) \right) \quad (28)$$

$$v_{Sb} = \eta_3 \left(v_{maxSb} \left(\frac{Sg}{kSgSb+Sg} \right) \left(1 - \left(\frac{But}{kBut} \right) \right) \right) - \frac{\eta_2 \left(v_{maxBut} * \left(\frac{Sb}{kSb+Sb} \right) \right)}{Y_{ButSb}} \quad (29)$$

$$v_{Ace} = \eta_4 \left(v_{maxAce} \left(\frac{Act}{kAA+Act} \right) \right) \quad (30)$$

$$v_{Act} = \eta_5 \left(v_{maxAct} \left(\frac{Sg}{kSgAct+Sg} \right) \left(\frac{1}{1 + \left(\frac{Ace}{kiAce} \right)} \right) \right) - \frac{\eta_4 \left(v_{maxAce} * \left(\frac{Act}{kAA+Act} \right) \right)}{Y_{AceAct}} \quad (31)$$

$$v_{Et} = \eta_6 \left(v_{maxEt} \left(\frac{Sg}{kSgEt+Sg} \right) \right) \quad (32)$$

$$\frac{dSg}{dt} = D(Sg_{in} - Sg) - \left(\frac{\mu_X \cdot X}{Y_{X/Sg}} \right) \quad (33)$$

$$\frac{dX}{dt} = -\psi DX + (\mu_X - k_{spo})X \quad (34)$$

$$\frac{dBut}{dt} = -D(But) + v_{But}X \quad (35)$$

$$\frac{dSb}{dt} = D(Sb_{in} - Sb) + v_{Sb}X \quad (36)$$

$$\frac{dAct}{dt} = -D(Act) + v_{Act}X \quad (37)$$

$$\frac{dAce}{dt} = -D(Ace) + v_{Ace}X \quad (38)$$

$$\frac{dEt}{dt} = -D(Et) + v_{Et}X \quad (39)$$

En el caso de la caracterización del sistema que opera con células inmovilizadas, se consideró un enfoque pseudo-homogéneo para caracterizar el efecto que las limitaciones de difusión tienen sobre el sistema en función de la aplicación del llamado factor de eficiencia (η_i). Este parámetro se define como el cociente de la velocidad de reacción aparente considerando las restricciones de difusión entre la velocidad de reacción observada en un sistema sin limitación por transferencia de masa, por lo que puede tomar valores entre 0 y 1 (Rout y Jakobsen, 2012).

Finalmente, para considerar el hecho de que la biomasa dentro del reactor en el sistema celular inmovilizado está unida al soporte y está suspendida dentro del reactor, este fenómeno se considera incluyendo un parámetro adicional ψ en el balance de masa para la concentración de biomasa. Este parámetro representa la fracción de masa de biomasa que sale del reactor debido al flujo de masa de entrada-salida en régimen continuo. El valor de ψ puede variar de 0 a 1, donde $\psi = 0$ representa la dinámica de flujo de lecho fijo y $\psi = 1$ representa el caso ideal del reactor continuo de "mezcla perfecta" (Méndez-Acosta et al., 2005).

En cuanto a la inclusión de los parámetros pseudo-homogéneos en el modelo estructurado, se debe mencionar que los ODE representan tanto los productos intracelulares como los extracelulares como parte del mismo "compartimento" (es decir, no existe una discretización explícita de los contenidos de la célula y el contenido del medio de cultivo), la asignación de factores de eficiencia a las velocidades intracelulares parece inadecuada, ya que la hipótesis es que la limitación de transferencia de masa descrita para dichos parámetros solo afecta el flujo de moléculas hacia y desde la célula, no dentro eso; tal consideración y también el hecho de que cada compuesto extracelular se correlaciona directamente con un metabolito intracelular en las ecuaciones seleccionadas limita su uso a sistemas de células libres solamente.

Identificación paramétrica de los modelos construidos

Los Cuadros 3, 4 y 5 resumen los valores obtenidos por la identificación paramétrica obtenida para los modelos tradicional, fenomenológico y estructurado, respectivamente. La validación de los modelos seleccionados se realizó mediante simulación numérica considerando las siguientes condiciones iniciales: $X_0 = 0.2 \text{ g L}^{-1}$, $Sg_0 = 60 \text{ g L}^{-1}$, $But_0 = 0.01 \text{ g L}^{-1}$, $Sb_0 = 0.01 \text{ g L}^{-1}$, $Ace_0 = 0.01 \text{ g L}^{-1}$, $Act_0 = 0.01 \text{ g L}^{-1}$ y $Et_0 = 0.01 \text{ g L}^{-1}$ (Figura 5). Cabe señalar que a los parámetros η_i y ψ se les asigna un valor igual a uno cuando se utiliza la estructura para modelar el sistema con celdas libres, tanto en régimen discontinuo como

continuo. Además, el rendimiento de las variables intracelulares descritas por el modelo estructurado se presenta en la Figura 6, donde debe observarse que todos los metabolitos modelados exhiben un comportamiento estable.

Cuadro 3. Identificación paramétrica del modelo No Estructurado Tradicional Ludeking-Piret considerando datos experimentales de un sistema de fermentación ABE que opera con células libres reportado por Yen y Li (2011).

Parámetro	Valor	Unidad	Parámetro	Valor	Unidad
α_{Ace}	0.1934 ± 0.4552	g g ⁻¹	β_{Sb}	-0.04 ± 0.0499	g h ⁻¹
α_{Act}	0.4737 ± 0.3788	g g ⁻¹	k_{But}	11.5657 ± 2.5350	g L ⁻¹
α_{But}	0.8712 ± 0.7405	g g ⁻¹	k_{Spo}	0.0834 ± 0.0646	g L ⁻¹
α_{Et}	0.0185 ± 0.36	g g ⁻¹	k_{Sg}	19.5091 ± 34.5349	g L ⁻¹
α_{Sb}	0.4951 ± 0.3798	g L ⁻¹	k_{Si}	262.5 ± 63.197	g L ⁻¹
β_{Ace}	0.1 ± 0.0620	g h ⁻¹	μ_{maxX}	0.4759 ± 0.4133	h ⁻¹
β_{Act}	-0.0354 ± 0.0493	g h ⁻¹	Y_{XSg}	0.085 ± 0.0377	g g ⁻¹
β_{But}	0.1364 ± 0.0725	g h ⁻¹	ψ	1	----
β_{Et}	0.0386 ± 0.0497	g h ⁻¹	η_1	1	----

Cuadro 4. Identificación paramétrica del modelo No Estructurado Fenomenológico considerando datos experimentales de un sistema de fermentación ABE que opera con células libres reportado por Yen y Li (2011).

Parámetro	Valor	Unidad	Parámetro	Valor	Unidad
k_{AA}	3.7924 ± 1.1382	$g L^{-1}$	V_{maxEt}	0.0368 ± 0.044	h^{-1}
k_{But}	9.8772 ± 1.1608	$g L^{-1}$	V_{maxSb}	0.7159 ± 0.0758	h^{-1}
k_{Spo}	0.1 ± 0.0590	h^{-1}	μ_{maxX}	0.6003 ± 0.0738	h^{-1}
k_{iAce}	16.4454 ± 6.4519	$g L^{-1}$	Y_{AceAct}	0.4182 ± 0.1699	$g g^{-1}$
k_{Sb}	3.5 ± 3.0195	$g L^{-1}$	Y_{ButSb}	0.4852 ± 0.0645	$g g^{-1}$
k_{Sg}	25 ± 4.9228	$g L^{-1}$	Y_{XSg}	0.1088 ± 0.0403	$g g^{-1}$
k_{SgAct}	0.5 ± 0.0684	$g L^{-1}$	ψ	1	----
k_{SgEt}	0.0726 ± 0.0018	$g L^{-1}$	η_1	1	----
k_{SgSb}	2.51 ± 0.9926	$g L^{-1}$	η_2	1	----
k_{Si}	281.9999 ± 6.1425	$g L^{-1}$	η_3	1	----
V_{maxAce}	0.6361 ± 0.1581	h^{-1}	η_4	1	----
V_{maxAct}	0.3349 ± 0.1079	h^{-1}	η_5	1	----
V_{maxBut}	0.9949 ± 0.0684	h^{-1}	η_6	1	----

Cuadro 5. Identificación paramétrica del modelo estructurado considerando datos experimentales de un sistema de fermentación ABE que opera con células libres reportado por Yen y Li (2011).

Parámetro	Valor	Unidad	Parámetro	Valor	Unidad
k_{But}	8.1600 ± 13.3063	g L ⁻¹	k_{Si}	251.7550 ± 62.3938	g L ⁻¹
k_{Spo}	0.09256 ± 0.1402	h ⁻¹	μ_{maxX}	0.5078 ± 0.2496	h ⁻¹
k_{m1}	9.9610 ± 1.0583	g L ⁻¹	V_{max1}	0.9929 ± 0.7247	h ⁻¹
k_{m2}	0.0103 ± 0.0905	g L ⁻¹	V_{max2}	0.9471 ± 0.8214	h ⁻¹
k_{m3}	0.0151 ± 0.0702	g L ⁻¹	V_{max3}	0.9732 ± 0.3080	h ⁻¹
k_{m4}	0.0279 ± 0.0392	g L ⁻¹	V_{max4}	0.0208 ± 0.0214	h ⁻¹
k_{m5}	0.9889 ± 0.1094	g L ⁻¹	V_{max5}	0.0101 ± 0.0096	h ⁻¹
k_{m6}	0.0122 ± 0.0698	g L ⁻¹	V_{max6}	0.9431 ± 0.4049	h ⁻¹
k_{m7}	0.5324 ± 0.5232	g L ⁻¹	V_{max7}	0.9692 ± 0.3387	h ⁻¹
k_{m8}	0.0030 ± 0.0035	g L ⁻¹	V_{max8}	0.2136 ± 0.1419	h ⁻¹
k_{m9}	0.0102 ± 0.0333	g L ⁻¹	V_{max9}	0.7668 ± 0.2779	h ⁻¹
k_{m10}	0.0118 ± 0.0387	g L ⁻¹	V_{max10}	0.8609 ± 0.3522	h ⁻¹
k_{m11}	0.0119 ± 0.0388	g L ⁻¹	V_{max11}	0.0769 ± 0.0313	h ⁻¹
k_{m14}	0.0540 ± 0.0624	g L ⁻¹	V_{max14}	0.8854 ± 0.9184	h ⁻¹

k_{m16}	$0.0109 \pm$ 0.0125	$g L^{-1}$	v_{max16}	0.1651 ± 0.2201	h^{-1}
k_{m17}	$0.1340 \pm$ 0.1442	$g L^{-1}$	v_{max17}	0.0141 ± 0.0683	h^{-1}
k_{m18}	$0.0102 \pm$ 0.0232	$g L^{-1}$	v_{max18}	0.0675 ± 0.0170	h^{-1}
k_{m19}	$0.0245 \pm$ 0.0131	$g L^{-1}$	v_{max19}	0.9869 ± 0.5956	h^{-1}
k_{Sg}	$24.7464 \pm$ 3.0122	$g L^{-1}$	Y_{XSg}	0.1000 ± 0.2344	$g g^{-1}$

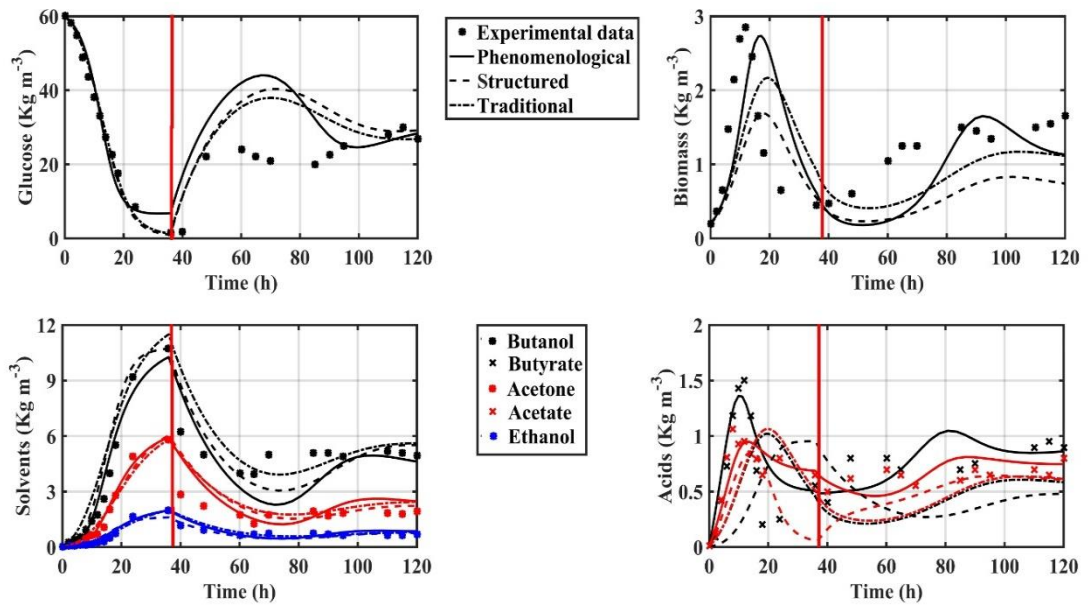


Figura 5. Validación de los modelos cinéticos seleccionados frente a datos experimentales de *Clostridium acetobutylicum* ATCC 824 en un reactor que opera con células libres reportados por Yen y Li (2011). La línea horizontal indica el cambio entre la operación por lote y el régimen continuo (36 h).

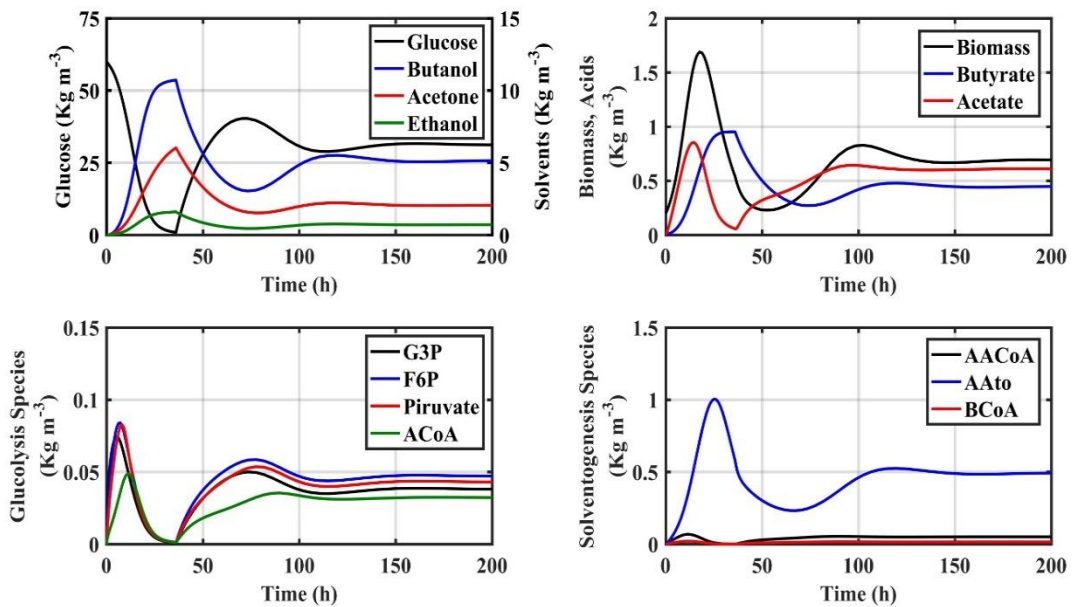


Figura 6. Resultados de la simulación de las variables intracelulares modeladas utilizando el modelo cinético estructurado construido en este trabajo para *Clostridium acetobutylicum* ATCC 824. La línea horizontal indica el cambio entre lote y régimen continuo (36 h).

La alta incertidumbre paramétrica observada en algunos valores estimados se puede atribuir al hecho de que los datos experimentales se miden con precisión finita y solo un subconjunto de las variables de estado se puede acceder experimentalmente de forma "en línea", ya que las muestras se deben obtener del cultivo y procesado fuera de línea, esto, junto con la naturaleza de no linealidad inherente de los sistemas biológicos pueden ser las principales causas de tales resultados (Vanlier et al., 2013).

Para los parámetros obtenidos se debe tener en cuenta que los tres valores obtenidos para μ_{maxX} , k_{But} , k_{Si} , k_{Sg} y Y_{XSg} son todos estadísticamente iguales en función de los intervalos de confianza determinados por el algoritmo de Marquardt, que es un comportamiento esperado considerando el hecho de que la tasa de crecimiento las cinéticas están representadas por la misma estructura en todos los modelos probados. Además, todos los valores de los parámetros mencionados anteriormente se encuentran dentro de las regiones reportadas en la literatura para la bacteria *Clostridium* (Procentese et al., 2013; Raganati et al., 2015).

Para evaluar las capacidades descriptivas de los tres modelos se realizó un cálculo de bondad de ajuste utilizando el procedimiento de coeficiente de determinación clásico y también por el Π índice de rendimiento de modelado, cuyos resultados se presentan en el Cuadro 4. Debe ser necesario recordar que un valor negativo el valor para R^2 indica que el rendimiento de ajuste del modelo frente a los datos experimentales es peor que el que usa una sola línea horizontal con un valor igual al promedio del conjunto de datos, y tal condición indicaría que el modelo seleccionado no es adecuado para representar el comportamiento del sistema. Además, en principio los tres modelos matemáticos logran un índice de determinación global $R^2 > 0.95$ y también un valor de $p < 0.001$, lo que asegura que no hay una diferencia significativa entre el comportamiento predicho descrito por el modelo propuesto y los datos experimentales (Selke et al., 2001).

Cuadro 6. Índices de rendimiento estadístico de los modelos cinéticos evaluados considerando los datos experimentales obtenidos bajo el régimen de lote con células libres reportados por Yen y Li (2011).

Variable	Tradicional		Fenomenológico		Estructurado	
	R^2	Π	R^2	Π	R^2	Π
Glucosa	0.9773	0.8723	0.9721	0.8568	0.9869	0.9030
Biomasa	-0.0209	-0.1481	0.1277	0.1943	0.0793	-0.2110
Butanol	0.9632	0.8108	0.9729	0.8413	0.9163	0.7693
Butirato	-0.2788	-0.2620	0.8117	0.5319	-1.1006	-0.2869
Acetato	-0.2886	0.1913	0.7735	0.5626	0.0892	0.2565
Acetona	0.9693	0.8304	0.9840	0.8859	0.9733	0.8461
Etanol	0.9619	0.8344	0.9511	0.8179	0.8902	0.6329
Global	0.9732	0.8532	0.9630	0.8619	0.9807	0.8397

Una nota interesante sobre los resultados de este análisis es la amplia brecha entre el rendimiento de modelado de las estructuras, particularmente en la aproximación de la dinámica de la biomasa y de los ácidos orgánicos. El primer fenómeno puede atribuirse a la incapacidad de la velocidad cinética de crecimiento seleccionada para ofrecer una representación precisa de la fase de crecimiento exponencial del cultivo, ya que los tres modelos indican que el cultivo comienza dicha etapa metabólica después de 6 a 8 horas de fermentación, mientras que los datos experimentales indican claramente que el inóculo se alimentó en condiciones óptimas para comenzar la propagación celular tan pronto como comenzó la fermentación.

Por el contrario, por el bajo rendimiento de los modelos tradicional y estructurado con respecto al comportamiento con ácidos orgánicos, el primero tiene la desventaja de representar variables tales como la dependencia lineal del crecimiento y la cantidad celular en forma de los parámetros α y β de Ludeking-Piret, y si bien los valores obtenidos tienen sentido a partir de la concepción del modelo original para la generación de metabolitos, porque el valor α positivo y

negativo β pueden interpretarse como una representación básica de la fase acidogénica y solventogénica de la fermentación, respectivamente, es precisamente el hecho de que β toma un valor negativo que hace que la curva modelada represente una concentración de ácido inferior a la experimental, incluso decayendo a valores negativos en tiempos de simulación largos (> 300 h). En el otro escenario, el modelo estructurado adolece de un índice de determinación bajo para la reproducción de acetato y butirato debido a que los metabolitos explícitamente mencionados en sus ecuaciones de balance de masa son especies intracelulares que 1) tienen una tasa de interconversión alta que los hace comportarse como variables pseudo-estacionarias y 2) la determinación experimental de la concentración de tales moléculas en tiempo real no se considera en los valores informados para la identificación paramétrica por ser especies intracelulares.

Con este ejercicio, debe quedar claro que orientar la selección de un modelo cinético solo considerando un índice de determinación global no es aconsejable, especialmente si el objetivo de la construcción del modelo es utilizarlo como base para la aplicación de análisis, diseño e intensificación de bioprocesos. Tal cuestión condujo al siguiente análisis, donde el rendimiento de los tres modelos evaluados ahora se determinó calculando su bondad de ajuste fuera de la región operativa en la que se ajustaron, con la hipótesis de que si tanto el tipo de reactor, el régimen operativo, utilizaba la cepa y el medio de cultivo utilizado no cambia, entonces el metabolismo interno de la célula no debería verse afectado. Esta hipótesis es vital en la determinación de las capacidades predictivas de cualquier modelo dado, ya que tradicionalmente hay una amplia gama de trabajos informados en la literatura donde los autores hacen uso de una metodología iterativa para identificación paramétrica y cambian todos los valores de los parámetros para hacer sus estructuras ajustar los datos en diferentes condiciones de proceso, incluso si todas las variables operacionales anteriores permanecen constantes (López y Borzacconi, 2010; López et al., 2015).

Comparación de desempeño entre modelos

Para tal ejercicio se consideraron los datos experimentales en régimen de lote a diferentes concentraciones iniciales de glucosa informadas por Qureshi et al. (2007), donde el objetivo, aparte de la meta mencionada en el párrafo anterior, era corroborar que las estructuras analizadas eran capaces de reproducir el efecto de inhibición del crecimiento informado tanto en los trabajos de Qureshi et al. (2007) y Lee et al. (2007), ya que este paso es crucial para determinar si los modelos se pueden usar al menos para diseñar y optimizar las condiciones para el cultivo por lotes con celdas libres. Los resultados del análisis se muestran en la Figura 7, donde el coeficiente de determinación después del nombre del modelo representa su bondad de ajuste para el rendimiento general frente al cambio de las condiciones operacionales y los cuadros sobre las barras ayudan a comparar la significación estadística entre los valores experimentales (en barras negras) y los valores predichos de los tres modelos, donde las áreas de recuadro superpuestas indican la equivalencia estadística entre ellos.

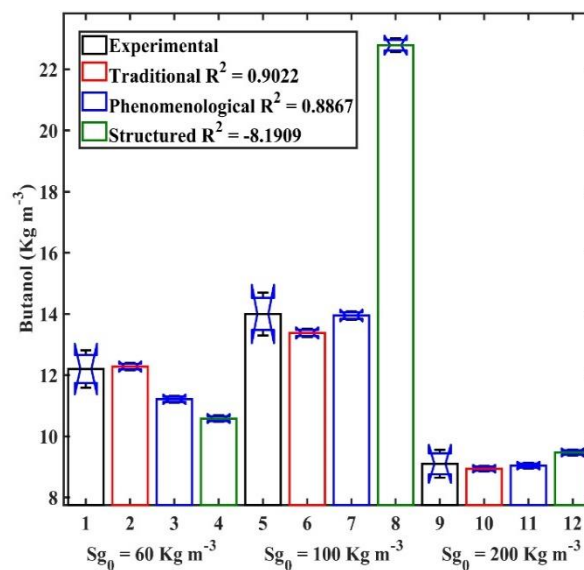


Figura 7. Comparación estadística entre datos experimentales de Qureshi et al. (2007) y valores de simulación obtenidos por los modelos cinéticos seleccionados para *Clostridium acetobutylicum* ATCC 824 bajo operación discontinua con células libres a diferentes concentraciones iniciales de glucosa.

Teniendo en cuenta los datos obtenidos de la Figura 7 es evidente que el modelo estructurado no es adecuado para representar el comportamiento real de la producción de butanol sobre las condiciones experimentales probadas, ya que incluso si es capaz de reproducir el efecto de inhibición del sustrato sobre la fermentación, los valores que genera una diferencia estadísticamente significativa para las tres condiciones iniciales de glucosa. Dichos resultados pueden considerarse contradictorios debido a la hipótesis de que un modelo más complejo construido bajo un conocimiento más amplio de la vía metabólica debería conducir a una mejor representación del sistema; sin embargo, como se mencionó anteriormente, el bajo rendimiento puede atribuirse a la limitada información experimental disponible para la identificación paramétrica del modelo estructurado.

Una vez descartado el uso del modelo Estructurado para posteriores análisis se procedió al análisis de desempeño de las dos estructuras restantes contra datos experimentales obtenidos con el uso de células inmovilizadas. Para este caso de estudio se reconsideró la hipótesis de que incluso si las células sufrirían una limitación de la transferencia de masa debido al uso de la matriz de inmovilización, su metabolismo intrínseco no cambiaría y que los únicos parámetros que se re-identificarían en tal escenario serían los índices de flujo no ideales y los factores de eficiencia indicados por ψ y η_i , respectivamente. Como una consideración adicional, debido a los informes en la literatura que atribuyen el aumento de la generación de alcohol a un efecto de protección contra la inhibición del producto tanto en ABE como en los sistemas productores de etanol, los parámetros k_{SpO} y k_{But} también se incluyeron en ese conjunto.

Con lo anterior, los Cuadros 7 y 8 indican los resultados de la identificación paramétrica del conjunto de parámetros inmovilizados en el límite mencionado en el párrafo anterior para los modelos tradicional y fenomenológico, respectivamente. La validación de los modelos elegidos bajo este régimen operacional se realizó mediante simulación numérica considerando las

condiciones iniciales reportadas por Yen y Li (2011): $X_0 = 0.5 \text{ g L}^{-1}$, $S_{g0} = 60 \text{ g L}^{-1}$, $But_0 = 0.01 \text{ g L}^{-1}$, $S_{b0} = 0.01 \text{ g L}^{-1}$, $Ace_0 = 0.01 \text{ g L}^{-1}$, $Act_0 = 0.01 \text{ g L}^{-1}$ y $E_{t0} = 0.01 \text{ g L}^{-1}$ (Figura 8), donde ambas estructuras alcanzan un índice de determinación global $R^2 > 0.95$ y un valor de $p < 0.001$.

Cuadro 7. Identificación paramétrica del modelo tradicional considerando los datos experimentales de un sistema de fermentación ABE que opera con células inmovilizadas reportado por Yen y Li (2011).

Parámetro	Valor	Unidad
k_{But}	21.1500 ± 3.2688	g L^{-1}
k_{Spo}	0.0718 ± 0.0086	h^{-1}
ψ	0.125	----
η_1	0.6268 ± 0.0211	----

Cuadro 8. Identificación paramétrica del modelo fenomenológico considerando los datos experimentales de un sistema de fermentación ABE que opera con células inmovilizadas reportado por Yen y Li (2011).

Parámetro	Valor	Unidad
k_{But}	17.1115 ± 3.5613	g L^{-1}
k_{Spo}	0.0671 ± 0.0176	h^{-1}
ψ	0.35	----
η_1	0.6196 ± 0.0333	----
η_2	0.5551 ± 0.1206	----
η_3	0.7843 ± 0.0843	----
η_4	0.6140 ± 0.1692	----
η_5	0.995 ± 0.1658	----
η_6	0.7110 ± 0.2926	----

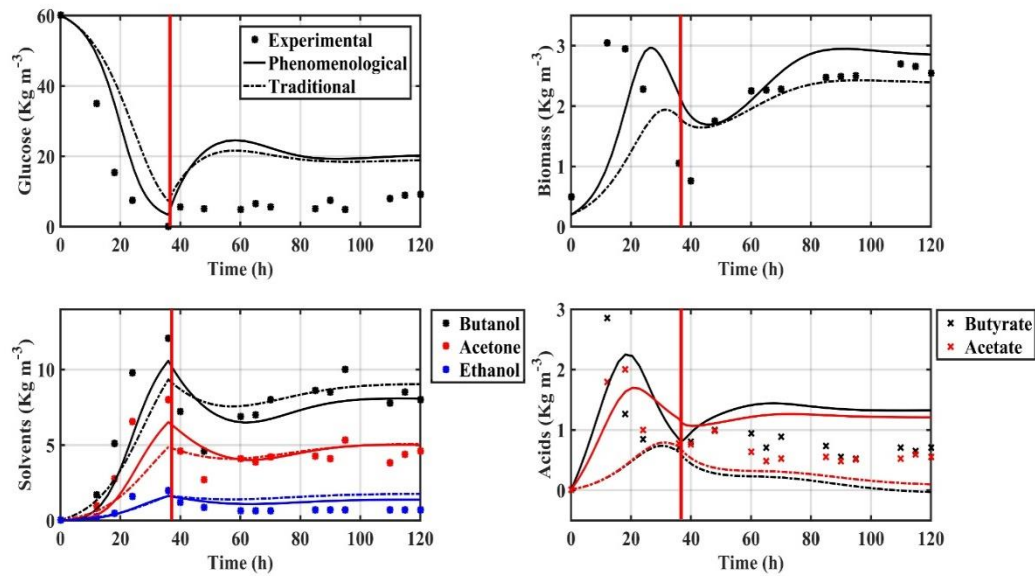


Figura 8. Validación de los modelos cinéticos seleccionados versus datos experimentales de *Clostridium acetobutylicum* ATCC 824 de un reactor que opera con células inmovilizadas reportadas por Yen y Li (2011). La línea horizontal indica el cambio entre el lote y el régimen continuo (36 h).

Tras la validación previa se realizó el cálculo de los coeficientes de determinación individuales para las siete variables modeladas y para ambos modelos evaluados, cuyos resultados mantienen el mismo comportamiento observado durante el cálculo de bondad de ajuste para el caso de célula libre: el modelo tradicional tiene dificultades para reproducir con precisión la dinámica de la producción y el consumo de butirato y acetato debido a un valor β negativo presente en esas ecuaciones, que también causa una disminución en la concentración prevista de esos ácidos por debajo de cero cuando el tiempo de simulación excede las 100 horas (Cuadro 9).

Cuadro 9. Índices de rendimiento estadístico de los modelos cinéticos evaluados considerando los datos experimentales obtenidos bajo el régimen de lote con células inmovilizadas reportadas por Yen y Li (2011).

Variable	Tradicional		Fenomenológico	
	R^2	Π	R^2	Π
Glucosa	0.9984	0.9692	0.9953	0.9478
Biomasa	0.8892	0.8070	0.9074	0.8343
Butanol	0.9580	0.8862	0.9928	0.9498
Butirato	0.2511	0.1957	0.8274	0.7331
Acetato	0.4428	0.2761	0.8883	0.7859
Acetona	0.9495	0.8299	0.9505	0.8662
Etanol	0.8580	1	0.9710	1
Global	0.9874	0.9035	0.9952	0.9418

Siguiendo la suposición previa de que la selección de una estructura adecuada para el uso en el análisis y diseño de bioprocesos no debe realizarse considerando únicamente los coeficientes de determinación de las estructuras en las mismas condiciones en que se ajustaron, se realizó un análisis estadístico comparando los resultados de la simulación numérica obtenida con ambos modelos versus datos experimentales contra un reactor de operación continua con variaciones en a) Tasa de dilución (D) (Figura 9) y b) Concentración de glucosa en la alimentación en la corriente de entrada ($S_{g_{in}}$) (Figura 10). Nuevamente, el coeficiente de determinación indicado para cada modelo es el que compara la bondad de ajuste del comportamiento modelado del sistema con la tendencia de datos experimentales junto con la variable operacional manipulada.

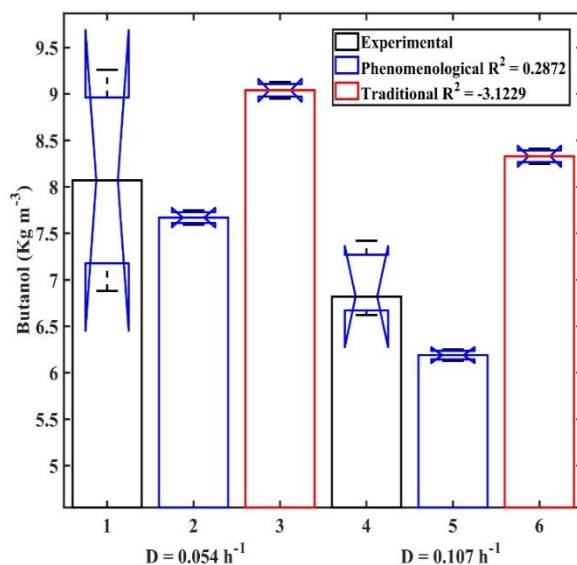


Figura 9. Comparación estadística entre los datos experimentales de butanol de Yen y Li (2011) y los valores de simulación obtenidos por los modelos cinéticos seleccionados para *Clostridium acetobutylicum* ATCC 824 en operación continua con células inmovilizadas y diferentes tasas de dilución (D). Las áreas de recuadro superpuestas indican la equivalencia estadística de los promedios de cada conjunto.

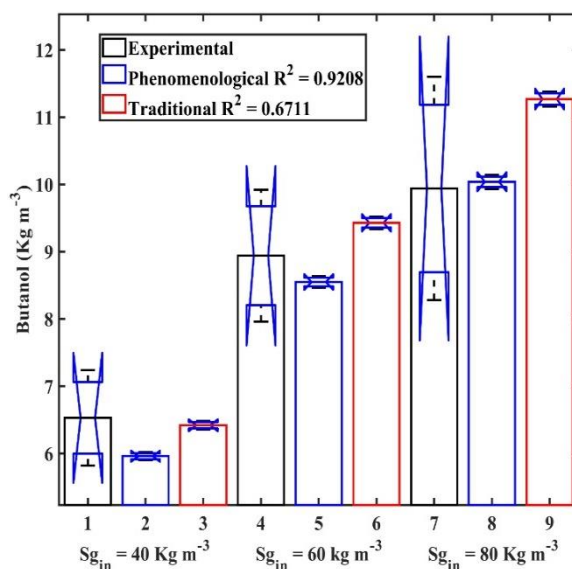


Figura 10. Comparación estadística entre datos experimentales de butanol de Yen y Li (2011) y valores de simulación obtenidos por los modelos cinéticos seleccionados para *Clostridium acetobutylicum* ATCC 824 en operación continua con células inmovilizadas y diferentes concentraciones de alimentación de glucosa (S_{gin}). Las áreas de recuadro superpuestas indican la equivalencia estadística de los promedios de cada conjunto.

Estos últimos resultados indican que el modelo fenomenológico tiene una mayor bondad de ajuste para los dos escenarios evaluados, incluso si el coeficiente de determinación obtenido en la Figura 10 es inferior a 0.95, como el valor negativo del coeficiente de determinación para el modelo tradicional junto con el hecho que el ensayo ANOVA aplicado para la comparación entre los tratamientos individuales indicó que el valor de simulación obtenido con el modelo fenomenológico es estadísticamente igual al experimental en condiciones operacionales de $D = 0.054 \text{ h}^{-1}$ y $S_{g_{in}} = 60 \text{ Kg m}^{-3}$ sugiere que tal la estructura de modelado tiene un potencial de aplicación más amplio en cuanto a su uso para predecir el rendimiento del sistema ABE bajo regímenes discontinuos y continuos para fermentaciones que operan con células inmovilizadas, manteniendo una estructura relativamente simple y haciendo uso de pocos parámetros adicionales para caracterizar tales condiciones.

Selección del régimen de operación

Referirse al **Anexo 1**: “Novel Kinetic Model for the Simulation Analysis of the Butanol Productivity of *Clostridium acetobutylicum* ATCC 824 under Different Reactor Configurations”.

Optimización del desempeño del sistema de fermentación ABE

Problema 1: Cultivo por lote.

En un trabajo anterior, Chang (2010) evaluó tanto teórica como experimentalmente el efecto de la adición de butirato en cultivos discontinuos de *Clostridium beijerinckii*, llegando a la conclusión que la suplementación del medio de cultivo utilizando concentraciones de butirato iniciales en un intervalo comprendido entre los 2 y los 5 g/L ofrecen un aumento de hasta 20 % en el título de butanol al final de la fermentación; asimismo demostró que valores superiores al intervalo mencionado anteriormente impactaban de manera negativa la

generación de butanol ya que el cultivo podría sufrir una escasez de ATP, con lo cual se veía reducido su crecimiento.

La Figura 11 muestra de manera visual el comportamiento de las variables a optimizar dentro del sistema dentro del rango de condiciones iniciales analizadas, rescatando que en este régimen no es posible determinar un solo máximo global para la concentración de butanol debido, sino que es posible obtener una curva de puntos máximos determinada por el “lomo” del semicilindro observado en la misma. Este comportamiento permite entonces involucrar como criterio adicional de selección del punto más conveniente la minimización de la concentración de sustrato requerida para alcanzar dicha curva, en el entendido de que el desempeño del sistema no se verá afectado de manera negativa. Asimismo es necesario recalcar que el cálculo de la productividad de butanol en este sistema se realizó considerando dividir la concentración obtenida en equilibrio cinético entre el tiempo que le tomó al sistema alcanzar dicha condición, misma que sin adición de butirato alcanza su mínimo a las 85 h iniciando la fermentación con 80 g/L de glucosa, sin embargo la adición de butirato en el sistema lo que provocó fue el desplazamiento de este punto mínimo hasta las 94.5 h con 70 g/L de glucosa inicial. Este fenómeno fue evidente desde la evaluación superficial del modo de operación realizada en la etapa anterior del proyecto.

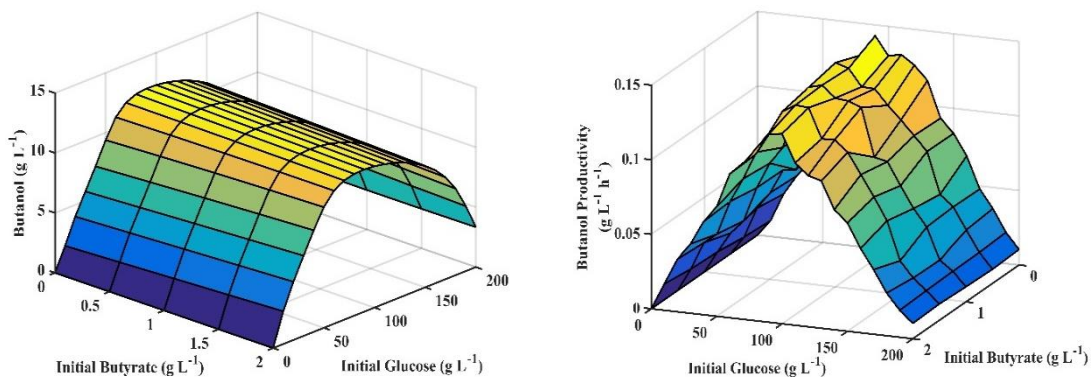


Figura 11. Comparación entre concentración de butanol (izquierda) y productividad del mismo (derecha) de *Clostridium acetobutylicum* ATCC 824 en régimen por lote con células libres con respecto a variaciones en condiciones iniciales de cultivo considerando S_{g0} y S_{b0} .

Por lo tanto, y determinar de manera oportuna las condiciones que maximizarían la productividad del sistema, lo cual se realizó considerando la concentración obtenida en equilibrio cinético dividido por el tiempo que tardó el sistema en alcanzar dicha condición, la formulación final para la optimización del problema de optimización para régimen por lote fue:

$$\max J_1 = But \quad (37)$$

$$\max J_2 = \frac{But}{t_f} \quad (38)$$

Sujeto a las siguientes restricciones:

Ecs. (30-36)

$$D = 0 \quad (39)$$

$$0 \leq Sg \leq 200 \quad (40)$$

$$0 \leq X \leq 10 \quad (41)$$

$$0 \leq But \leq 30 \quad (42)$$

$$0 \leq Sb \leq 20 \quad (43)$$

$$0 \leq Act \leq 20 \quad (44)$$

$$0 \leq Ace \leq 25 \quad (45)$$

$$0 \leq Et \leq 20 \quad (46)$$

$$80 \leq Sg_0 \leq 100 \quad (47)$$

$$0 \leq Sb_0 \leq 4 \quad (48)$$

$$12 \leq t_f \leq 48 \quad (49)$$

La Figura 12 contiene una muestra completa de los diferentes frentes de Pareto obtenidos para la resolución del problema 1, y como el umbral mínimo de concentración para garantizar la viabilidad técnica de los procesos posteriores es 12 g L^{-1} (Khöler et al., 2015), el punto operativo más adecuado está encapsulado en el círculo negro.

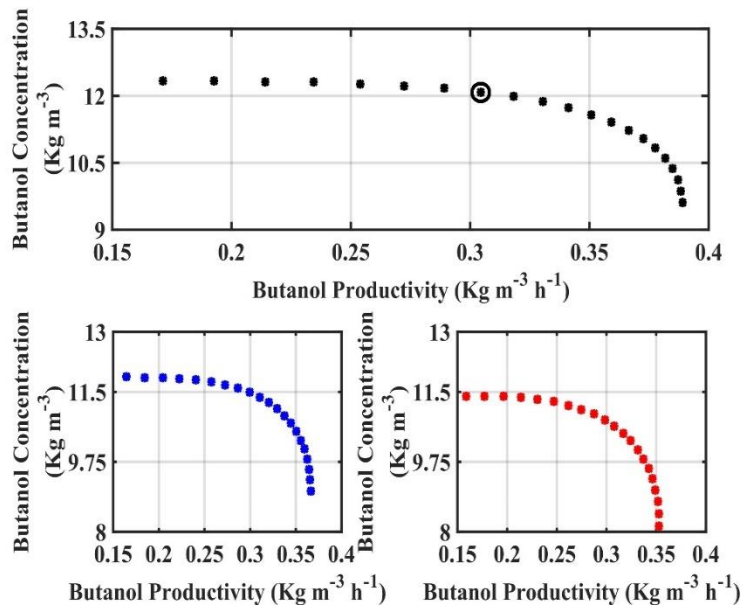


Figura 12. Frentes de Pareto para J1 (concentración de butanol) y J2 (productividad de butanol) del sistema de fermentación ABE operando bajo régimen de lotes con células libres considerando la manipulación de la glucosa inicial y el tiempo de fermentación terminal (Negro = Sin butirato inicial, Azul = Butirato inicial de 2 g L⁻¹ y rojo = butirato inicial de 4 g L⁻¹).

Las condiciones resultantes de este análisis fueron $S_{g0} = 90 \text{ g L}^{-1}$ y $S_{b0} = 0 \text{ g L}^{-1}$, que dan una concentración final de butanol de 12.043 g L^{-1} y una productividad de $0.3109 \text{ g L}^{-1} \text{ h}^{-1}$, respectivamente.

Problema 2: Fed-batch.

El régimen de operación semicontinuo es una técnica utilizada para reducir el impacto del fenómeno de inhibición por sustrato sobre un cultivo, generalmente se lleva a cabo iniciando con una fermentación por lote hasta que se logra ya sea una concentración de sustrato deseada o se alcanza una condición de equilibrio y a continuación, una solución con sustrato concentrado se alimenta a una velocidad constante, lineal, exponencial o intermitente. En la fermentación ABE, el cultivo por lote alimentado se recomienda sólo cuando existe la posibilidad de acoplar un proceso de recuperación de producto en línea (Ezeji et al. 2004; Qureshi et al. 2007), debido a los fuertes efectos de inhibición de butanol, un

simple fed-batch no tendría suficiente productividad para ofrecer alguna ventaja sobre el funcionamiento CSTR.

Con el fin de estudiar el comportamiento de un cultivo fed-batch, en la etapa anterior del proyecto se planteó un escenario teórico considerando un biorreactor de 1000 L de capacidad, iniciando operación por lote con la mitad de su volumen de operación ocupado y regímenes de flujo constante entre 2 y 10 L/h con una solución de glucosa a una concentración de 60 g/L. Sin embargo para este análisis se determinó que dichas condiciones eran demasiado restrictivas y que no reflejaban la realidad en cuanto al establecimiento de condiciones de operación necesarias para determinar la viabilidad de este sistema de fermentación en particular.

Además, uno de los problemas más recurrentes en el establecimiento de condiciones de operación óptimas para cultivos semicontinuos involucra la determinación de la política de alimentación del mismo, donde de manera tradicional se considera ya sea alimentación a flujo constante, lineal, exponencial o ligado a μ o a la generación de algún metabolito dentro de la reacción; sin embargo, la ventaja de poder utilizar algoritmos de optimización multiobjetivo es que es posible introducir la política de alimentación como parámetro a determinar para la maximización de los objetivos a analizar, con lo cual se pretende dar luz al respecto de tal problemática desde un punto de vista numérico. A su vez, no sólo la política de operación del reactor es de interés, sino también se consideró analizar el efecto del tiempo de inicio de la alimentación de sustrato en el sistema, considerando tres opciones distintas: iniciar una vez alcanzado el equilibrio cinético (36 h), al alcanzar la máxima concentración de biomasa (12 h) o desde el inicio de la fermentación como tal (0 h), considerando nuevamente que la política de alimentación no es constante e inicia siempre con un $F = 0$ L/h.

Por lo tanto, para la determinación de condiciones óptimas se consideró que como la política del flujo de alimentación del reactor es otro parámetro a manipular con respecto al tiempo de proceso se podría administrar una corriente

de alimentación más concentrada sin riesgo de inducir inhibición por sustrato, con lo cual se consideró una concentración de alimentación de glucosa de 200 g/L. Finalmente se consideró de manera adicional identificar la influencia de la suplementación del sistema con butirato, ya que no se evaluó su efecto en la etapa anterior.

Para esta finalidad se recurrió al uso del algoritmo de Programación Cuadrática Secuencial con restricciones, el cual se puede ejecutar desde la librería ACADOtoolkit, para lo cual se determinó que las restricciones del sistema, además del modelo cinético y de restricciones de factibilidad física (p. e.: $X > 0$ g/L; $V_0 < V < V_{total}$) se consideró que el tiempo de proceso no debería rebasar en total (fase de lote más fase semicontinua) las 50 horas, ya que más allá de este umbral de tiempo y asumiendo con base en la etapa previa que la concentración aproximada de butanol alcanzada en el sistema no rebasaría los 15 g/L la productividad del sistema sería menor que la de un cultivo por lote tradicional. Entonces, la formulación final para el problema de optimización de lotes alimentados fue:

$$\max J_1 = But \quad (50)$$

$$\max J_2 = \frac{But}{t_f} \quad (51)$$

Sujeto a las siguientes restricciones:

Ecs. (30-36)

$$0 \leq F \leq 25 \quad (52)$$

$$0 \leq Sg \leq 200 \quad (53)$$

$$0 \leq X \leq 10 \quad (54)$$

$$0 \leq But \leq 30 \quad (55)$$

$$0 \leq Sb \leq 20 \quad (56)$$

$$0 \leq Act \leq 20 \quad (57)$$

$$0 \leq Ace \leq 25 \quad (58)$$

$$0 \leq Et \leq 20 \quad (59)$$

$$0 \leq Sg_{in} \leq 200 \quad (60)$$

$$0 \leq Sb_{in} \leq 4 \quad (61)$$

$$750 \leq V \leq 1000 \quad (62)$$

$$10 \leq t_f \leq 50 \quad (63)$$

La Figura 13 muestra el frente de Pareto resultante considerando que el sistema opera con células libres y variando el tiempo inicial para el inicio de la alimentación del reactor sin suplementación de butirato, donde se puede ver que bajo las condiciones evaluadas se logra el mayor rendimiento del sistema al comenzar el flujo de entrada después de 12 h de cultivo discontinuo, entonces la concentración de biomasa en el reactor alcanza tanto su punto más alto como la tasa máxima de crecimiento. Bajo tal escenario, seleccionar el punto que maximiza J1 no es conveniente porque sacrifica casi el 50% de la productividad máxima, mientras que en el caso opuesto si se elige el punto que maximiza J2, la concentración final de producto en el reactor disminuye hasta 25 %, por lo tanto, se propone lograr un buen equilibrio entre los objetivos objetivo seleccionando el punto encapsulado en negro dentro del frente de Pareto, ya que la concentración del producto permanece por encima de 12 g L⁻¹ y la productividad aumenta de 0.1722 a 0.3295 g L⁻¹ h⁻¹. Tales resultados se corroboraron mediante simulación numérica y la dinámica resultante se describe en la Figura 14.

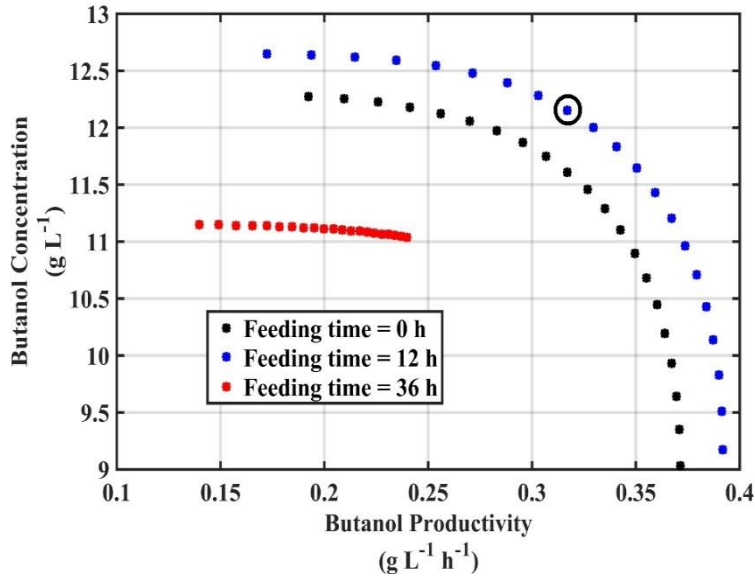


Figura 13. Frentes de Pareto para J1 (concentración de butanol) y J2 (productividad de butanol) del sistema de fermentación ABE que opera bajo régimen de lote alimentado con células libres considerando la manipulación de la glucosa inicial y el tiempo de fermentación terminal considerando diferentes tiempos iniciales para la activación de la alimentación escenario.

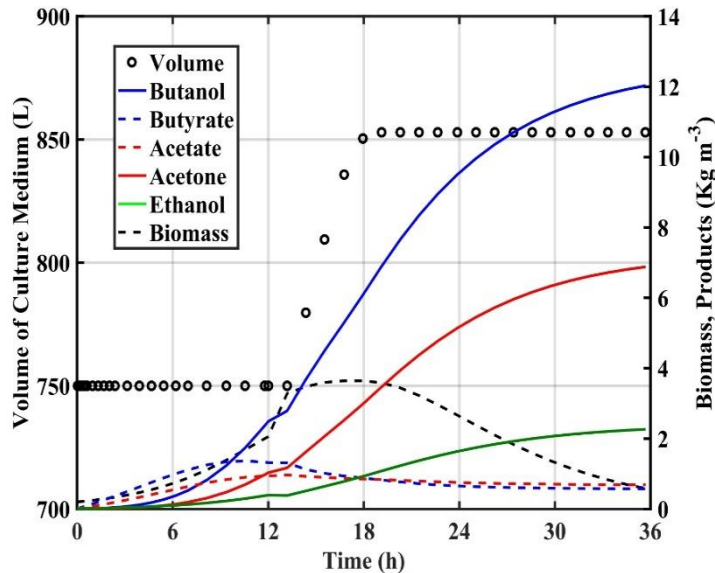


Figura 14. Simulación numérica del sistema de fermentación ABE que opera bajo régimen de lote alimentado con células libres considerando las condiciones del proceso obtenidas de la selección del punto de Pareto más adecuado de la Fig. 13 ($S_{ga} = 200 \text{ g L}^{-1}$, $S_{ba} = 0 \text{ g L}^{-1}$ y $t_f = 35,48 \text{ h}$).

Para este sistema de fermentación, considerando todas las variables analizadas en este trabajo, se encontró que el frente de Pareto con la mejor relación J_1 vs. J_2 fue el obtenido por el sistema operando con células inmovilizadas y comenzando la alimentación de entrada después de 12 h de lote régimen (Figura 15). Estas condiciones mostraron que para el caso de maximizar J_1 , se obtuvieron 20.01 g L^{-1} y $0.249 \text{ g L}^{-1} \text{ h}^{-1}$ y para el caso de maximizar J_2 , 12.3 g L^{-1} y $0.4388 \text{ g L}^{-1} \text{ h}^{-1}$ de concentración y productividad de butanol respectivamente.

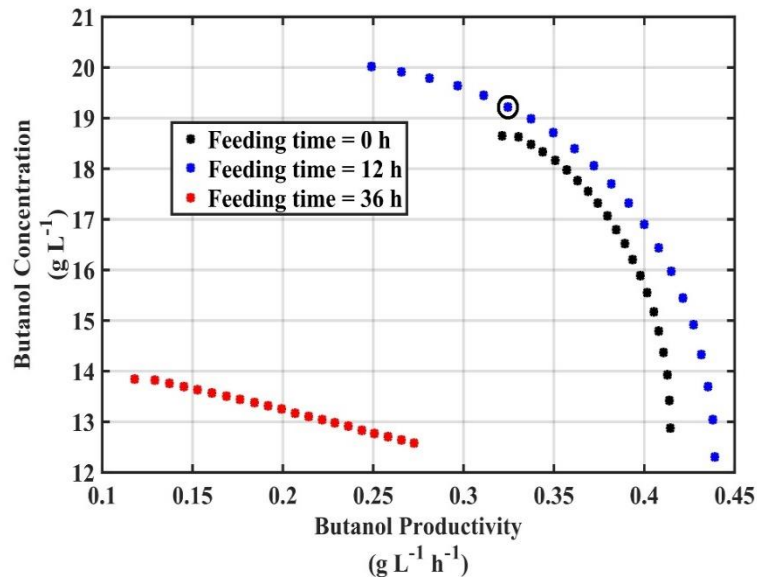


Figura 15. Frenes de Pareto para J_1 (concentración de butanol) y J_2 (productividad de butanol) del sistema de fermentación ABE que opera bajo régimen de lote alimentado con células inmovilizadas considerando la manipulación de la glucosa inicial y el tiempo de fermentación terminal considerando diferentes tiempos iniciales para la activación de la alimentación escenario.

Teniendo en cuenta los criterios expuestos por Khöler et al. (2015) sobre la relación lineal entre la concentración de butanol y la inversa del costo de recuperación de butanol dentro del rango de 7 a 19 g L^{-1} , el punto encerrado en el círculo negro se considera una elección razonable, teniendo en cuenta que el butanol concentración de 19.1454 g L^{-1} obtenida en tales condiciones frente a un cultivo discontinuo es superior al 50%. Además, debe tenerse en cuenta que el volumen del cultivo también aumentó de 750 a 987.62 L , lo que indica que no solo el producto está más concentrado sino que también tiene una mayor cantidad de masa en el recipiente de reacción (Figura 16).

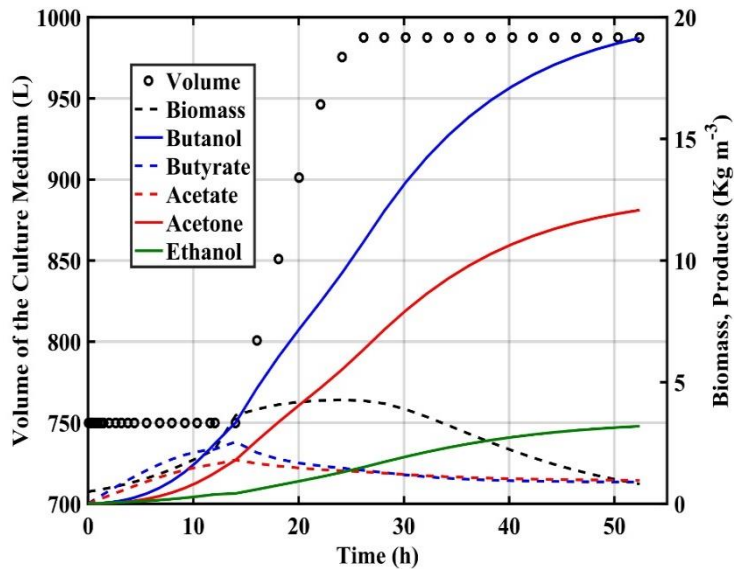


Figura 16. Simulación numérica del sistema de fermentación ABE que opera bajo régimen de lote alimentado con células inmobilizadas considerando las condiciones del proceso obtenidas de la selección del punto de Pareto más adecuado de la Fig. 15 ($S_{ga} = 200 \text{ g L}^{-1}$, $S_{ba} = 0 \text{ g L}^{-1}$ y $t_f = 52.39 \text{ h}$).

Problema 3: Régimen continuo.

Para la formulación del problema 3 se eliminó la necesidad de determinar el máximo de la productividad de butanol, incluso si dicha métrica es directamente proporcional a la tasa de dilución del sistema, la penalización sobre la concentración de butanol debido al lavado de las concentraciones internas dentro del reactor no es deseable debido a la economía mencionada anteriormente del proceso aguas abajo. Este efecto se refleja en las Figuras 17 y 18, que son los diagramas de bifurcación correspondiente para el sistema CSTR operando con células libres, donde se muestra que la concentración máxima butanol obtenido en tales condiciones no supera la marca de la 6 g L^{-1} . Además, estos diagramas ayudaron a complementar la formulación del problema 3 con información sobre la región más viable para encontrar el máximo numérico de las variables evaluadas, y también contribuyeron a inferir que el problema de optimización se encuentra dentro de una zona convexa que garantiza que los puntos óptimos encontrados numéricamente son únicos y globales (Nimmegeers et al., 2016).

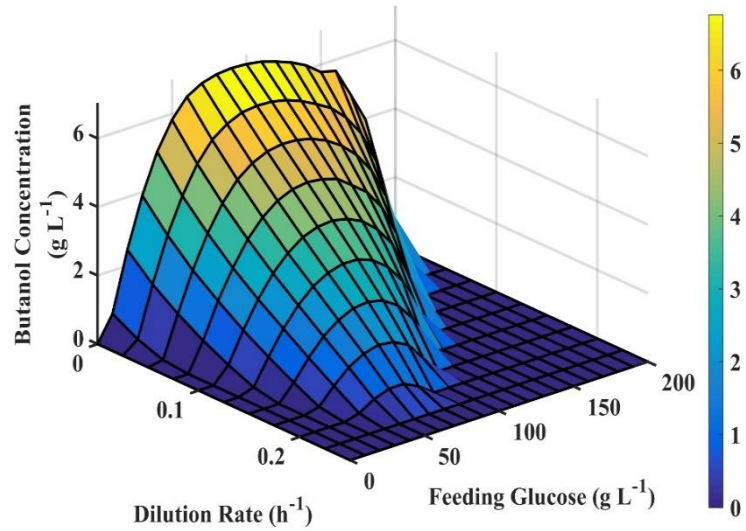


Figura 17. Diagrama de bifurcación del sistema de fermentación ABE de *Clostridium acetobutylicum* ATCC 824 con células libres que operan bajo el régimen CSTR con respecto a los cambios en la tasa de dilución (D) y la concentración de glucosa (S_{ga}) considerando la concentración de butanol como la variable de bifurcación.

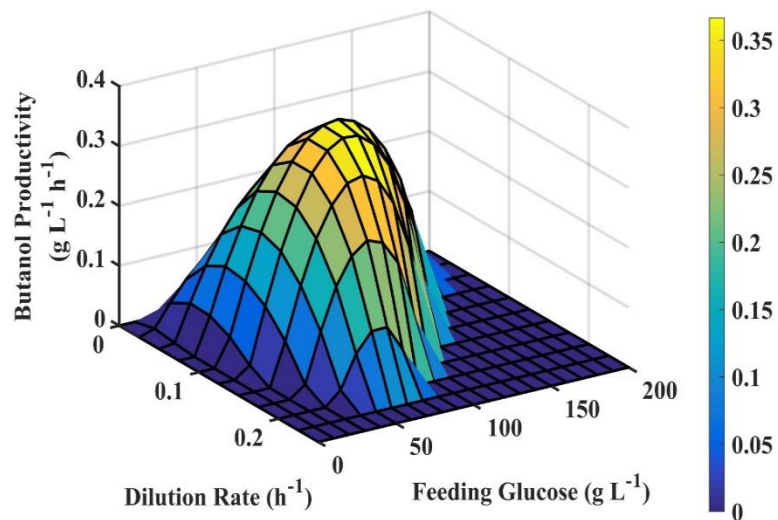


Figura 18. Diagrama de bifurcación del sistema de fermentación ABE de *Clostridium acetobutylicum* ATCC 824 con células libres que operan bajo el régimen CSTR con respecto a los cambios en la tasa de dilución (D) y la concentración de glucosa (S_{ga}) considerando la productividad de butanol como la variable de bifurcación.

Además, como la cepa *Clostridium acetobutylicum* utilizada tradicionalmente para la fermentación ABE sufre de inestabilidad genética debido a la pérdida del megaplasmido pSOL1, el tiempo del proceso se mantuvo en 500 h, ya que cualquier intervalo de tiempo superior sería inviable sin el uso de cepas genéticamente modificadas. Por lo tanto, la formulación del problema 3 dio como resultado lo siguiente:

$$\max J_1 = But \quad (64)$$

Sujeto a las siguientes restricciones:

Ecs. (30-36)

$$V = 750 \quad (65)$$

$$0 \leq Sg \leq 200 \quad (66)$$

$$0 \leq X \leq 20 \quad (67)$$

$$0 \leq But \leq 30 \quad (68)$$

$$0 \leq Sb \leq 20 \quad (69)$$

$$0 \leq Act \leq 20 \quad (70)$$

$$0 \leq Ace \leq 25 \quad (71)$$

$$0 \leq Et \leq 20 \quad (72)$$

$$90 \leq Sg_{in} \leq 120 \quad (73)$$

$$0 \leq Sb_{in} \leq 4 \quad (74)$$

$$0.025 \leq \frac{F}{V} = D \leq D_W = 0.45 \quad (75)$$

$$t_f = 500 \quad (76)$$

Análisis posteriores realizados con el sistema que opera con células inmovilizadas corroboraron la hipótesis expuesta en el segundo párrafo de esta sección, ya que se encontró que el punto más atractivo para este régimen alcanza una concentración de butanol de 10.07 g L⁻¹ y una productividad de 0.5036 g L⁻¹ h⁻¹, que es aproximadamente el mismo rendimiento para la concentración del

producto de un sistema por lote pero a una productividad mucho más alta (Figura 19).

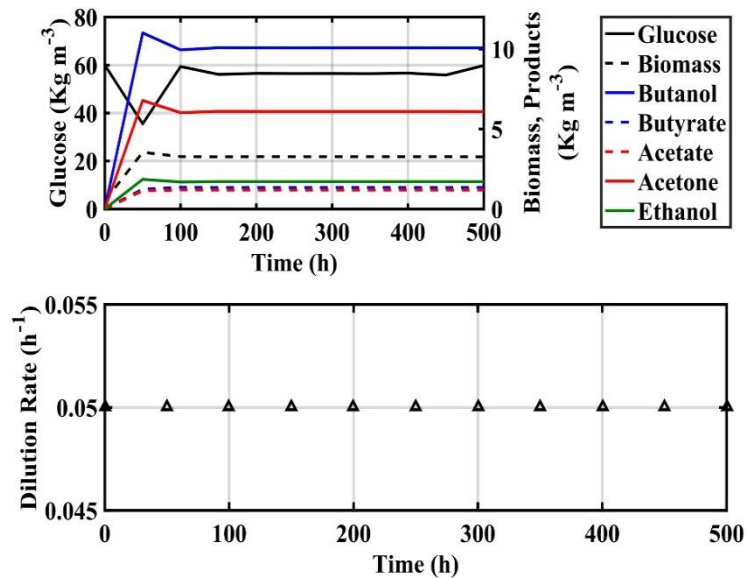


Figura 19. Perfil óptimo para la tasa de dilución (D) del sistema de fermentación ABE con células inmovilizadas que operan bajo el régimen CSTR para la maximización de la concentración de butanol.

Para completar el análisis, una tabla comparativa del desempeño de los regímenes operativos evaluados contra las métricas obtenidas por el sistema de fermentación comercial reportado por Jiang et al. (2015), donde se puede observar que el uso de reactores con células inmovilizadas puede ser una alternativa de aplicación para mejorar dicho proceso industrial.

Cuadro 10. Comparación de rendimiento entre un sistema industrial de fermentación ABE versus resultados de simulación obtenidos en este trabajo a través de simulación numérica bajo diferentes regímenes operacionales.

Configuración de reactor	Condiciones operativas	Concentración de butanol (g L⁻¹)	Productividad de butanol (g L⁻¹ h⁻¹)
Fed-Batch (Jiang et al., 2015)	Sg ₀ = 60 g L ⁻¹ t = 72 h	12.95	0.1799
Lote con células libres	Sg ₀ = 90 g L ⁻¹ Sb ₀ = 0 g L ⁻¹ t = 35.73 h	12.0431	0.3109
Fed-Batch con células libres	Sg ₀ = 60 g L ⁻¹ Sb ₀ = 0 g L ⁻¹ Sg _a = 200 g L ⁻¹ Sb _a = 0 g L ⁻¹ t = 35.48 h	12.0213	0.3388
Fed-Batch con células inmovilizadas	Sg ₀ = 60 g L ⁻¹ Sb ₀ = 0 g L ⁻¹ Sg _a = 200 g L ⁻¹ Sb _a = 0 g L ⁻¹ t = 52.39 h	19.1454	0.3655
Continuo con células libres	Sg ₀ = 60 g L ⁻¹ Sb ₀ = 0 g L ⁻¹ Sg _a = 96.81 g L ⁻¹ Sb _a = 0 g L ⁻¹ D = 0.025 h ⁻¹	6.0320	0.1508
Continuo con células inmovilizadas	Sg ₀ = 60 g L ⁻¹ Sb ₀ = 0 g L ⁻¹ Sg _a = 104.85 g L ⁻¹ Sb _a = 0 g L ⁻¹ D = 0.05 h ⁻¹	10.0711	0.5036

Referirse al **Anexo 2**: “Dynamic Nonlinear Feedback Control Applied to Improve Butanol Production by *Clostridium acetobutylicum*.”

Conclusiones

Se puede conseguir una buena representación cinética ($R^2 = 0.98$) de un proceso ABE realizado por lote con *Clostridium* inmovilizado en matriz porosa y utilizando glucosa como fuente de carbono mediante el uso de un modelo No Estructurado Fenomenológico y una parametrización simple de las limitaciones difusionales en forma de eficiencias de reacción y de retención de biomasa.

En este estudio de simulación se confirmó que un cultivo discontinuo alimentado con velocidad de flujo constante no es una estrategia de proceso ideal para mejorar la concentración y productividad butanol en fermentaciones ABE con respecto a sistemas por lote, ya que es superado por CSTR de una sola etapa en al menos un 20 y 300 % respectivamente. También se ha demostrado que el desarrollo de procesos fermentativos que involucren sistemas con inmovilización de biomasa, al menos para el cultivo evaluado en este trabajo, producen un impacto muy positivo sobre el título de butanol y la productividad del sistema, obteniendo valores superiores en 20.1544 y 591.94 % en comparación con el proceso industrial más reciente reportado hasta la fecha (Jiang et al., 2015).

Se propone el diseño y aplicación de un controlador sigmoide para mejorar la producción de butanol en un biorreactor continuo anaeróbico, para aumentar su productividad hasta un 56% en condiciones de alta alimentación de sustrato comparado con el sistema industrial tradicional en régimen semicontinuo reportado por Jiang et al. (2015). El controlador propuesto proporciona robustez al sistema de fermentación ABE frente a perturbaciones externas, y le confiere un comportamiento estable durante largos períodos de tiempo. Mediante experimentos numéricos, se demostró que la estructura de retroalimentación

propuesta proporciona un mejor rendimiento en comparación con el control PI clásico. Además, el controlador permite que el sistema converja a punto de funcionamiento estable con alta productividad en un tiempo de asentamiento bajo.

Perspectivas

Uno de los grandes retos pendientes en el estudio y mejoramiento del sistema de producción de butanol por medio de la fermentación ABE es la determinación de la política óptima de operación con respecto al pH del medio de cultivo, ya que como se mencionó en la subsección “*Ruta metabólica de la fermentación ABE*” la cascada de señalización para la producción de solventes en las cepas de *Clostridium spp.* están reguladas por la variación de la concentración de protones libres entre su membrana y pared celular. Por esta cualidad es necesario entonces profundizar en la correcta caracterización de los mecanismos que gobiernan dichos fenómenos metabólicos para poder incorporar al pH como variable de operación y optimización de la fermentación en términos explícitos. En el grupo de trabajo se han realizado ya las primeras aproximaciones para lograr esa meta, mismas que están plasmadas en el Anexo 3.

Asimismo, durante el desarrollo de este trabajo se pudo constatar que la implementación de técnicas de intensificación de procesos no está limitada al sistema de fermentación ABE estudiado para la presente tesis, sino que con el planteamiento adecuado de la representación cinética del proceso se puede adaptar la misma metodología para el mejoramiento de una gran variedad de procesos biológicos, tales como la producción de otro tipo de biocombustibles como lo es el caso del hidrógeno. Para esto ya se han realizado avances en la materia, mismos que se encuentran detallados en el Anexo 4.

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Anexo 1: "Novel Kinetic Model for the Simulation Analysis of the Butanol Productivity of *Clostridium acetobutylicum* ATCC 824 under Different Reactor Configurations".

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Article

Novel kinetic model for the simulation analysis of the butanol productivity of *Clostridium acetobutylicum* ATCC 824 under different reactor configurations



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ABSTRACT

Acetone–butanol–ethanol (ABE) fermentation process can be exploited for the generation of butanol as biofuel, however it does need to overcome its low volumetric solvent productivity before it can commercially compete with fossil fuel technologies. In this regard, mathematical modelling and simulation analysis are tools that can serve as the base for process engineering development of biological systems. In this work, a novel phenomenological kinetic model of *Clostridium acetobutylicum* ATCC 824 was considered as a benchmark system to evaluate the behaviour of an ABE fermentation under different process configurations using both free and immobilized cells: single stage batch operation, fed-batch, single stage Continuous Stirred Tank Reactor (CSTR) and multistage CSTRs with and without biomass recirculation. The proposed model achieved a linear correlation index $r^2 = 0.9952$ and $r^2 = 0.9710$ over experimental data for free and immobilized cells respectively. The predicted maximum butanol concentration and productivity obtained were $13.08 \text{ g}\cdot\text{L}^{-1}$ and $1.9620 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ respectively, which represents an increase of 1.01% and 990% versus the currently developed industrial scale process reported currently into the literature. These results provide a reliable platform for the design and optimization of the ABE fermentation system and showcase the adequate predictive nature of the proposed model.
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1. Introduction

Biofuels, which can be defined as all compounds of organic nature derived from living beings and their metabolism that can potentially be utilized as fuels, lead a group of alternative energy sources aimed to provide solution to the environmental issues caused by the overexploitation of fossil ones [1,2]. One of the most studied biofuels in the last decade is butanol, considering that it can offer better properties regarding fuel mileage yield, lower gaseous emissions, higher energy content and lower hygroscopicity versus the currently developed processes for the production of ethanol and biodiesel [3].

Traditionally, the methodology to obtaining butanol by fermentation is based on the degradation of various sugars (particularly glucose or sucrose) carried out by Gram-positive bacteria of the genus *Clostridium*, via a metabolic pathway called ABE (acetone–butanol–ethanol) [4]. ABE fermentation presents inherent restrictions that have prevented its consolidation as a mature technology such as low production yields, inhibitory effect over the culture's growth due to both solvents and glucose accumulation and mechanisms of metabolic regulation own of Gram-positive bacilli such as the sporulation process [5]. However,

ABE fermentation by *Clostridium* bacteria is still the most employed biological process for the solvent and biofuel production due to the poor performance obtained by heterologous recombinant strains such as *Escherichia coli*, which don't achieve butanol titers over $7 \text{ g}\cdot\text{L}^{-1}$ or volumetric productivities over $0.2 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ [2]. Furthermore, the petrochemical production of n-butanol is based on the hydrogenation of aldehydes obtained from the oxidation of propylene, making its production cost fluctuate alongside the crude oil market price [6].

One proposal that arises to overcome such challenges is the design and implementation of novel production strategies in order to find appropriate procedures to efficiently exploit the bacterial metabolism. From the above, traditional batch fermentations have been considered due their simple operating conditions and less prone to contamination, but the maximum historical butanol titre achieved in such configuration doesn't exceed $15 \text{ g}\cdot\text{L}^{-1}$ due inhibition of the bacterial growth. Continuous regime has the advantage to alleviate the negative side effects of both substrate and product accumulation, but the slow growth rate of *Clostridium* cultures does impede to operate under high dilution rates that can help to improve productivity of the system, while also causing that the final butanol titre at the outlet of the reactor dilute, which has a negative impact over downstream operation cost and energy consumption of the fermentation system [7]. Finally semi-continuous regimes applied to the ABE fermentation system had been successful

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only when they are coupled with *in situ* product recovery techniques, such as gas stripping or pervaporation. Nonetheless even if the experimental results show promising results towards product selectivity and culture growth maintenance there can be an opportunity to optimize the operational conditions in such complex systems to achieve lower costs and higher yields [8,9].

In this regard, mathematical modelling and simulation techniques can be used as the first step in novel process and bioreactor design, through the analysis of the behaviour of the culture under different operating conditions and regimes, where current literature has a wide variety of proposed structures for the description of the ABE fermentation process [10,11].

However, even considering that the mathematical modelling of the ABE fermentation system has at least 40 years of development, there is not a consensus about the most adequate structure to use in the scope of process design, control and optimization purposes, as the vast majority of reported model structures, they are only valid under specific process conditions and regimes. Recently, the so-called unstructured phenomenological kinetic modelling approach, sought to find the middle point between the complexity of metabolomic or genomic level models and easiness of use and interpretation of the traditional unstructured kinetic models, this approach has been successfully applied to the ABE system and is a solid prospect to evaluate the performance of this system under more complex regimes such as the ones involving medium optimization, multistage reactor clusters or integrative systems involving cell immobilization and *in situ* recovery strategies [20].

On other side, few information has been reported in the open literature regarding the effect of the operating regime in establishing ABE fermentation using *Clostridium* strains and the possible causes and effects associated to butanol production due to the use of one or another production strategy, as even experimental studies are generally limited to evaluate the performance of a particular operational regime under restrained conditions against classic batch methods, so many of the novel proposed processes are still open for analysis.

Therefore, in this work, a novel phenomenological unstructured kinetic model for ABE fermentation validated experimentally under batch and continuous operation is used to evaluate the theoretical butanol titre and productivity of a culture of *Clostridium acetobutylicum* ATCC 824 under the following four different production strategies: 1) adding butyric acid at the beginning of a batch fermentation, 2) single stage CSTR, 3) Three stage CSTRs with and without biomass recirculation and 4) fed-batch culture.

2. Methodology

2.1. Model development

A phenomenological non-structured kinetic model was developed to describe the kinetic behaviour of ABE production in batch reactor. The mathematical model is based on classical mass balance approach and it was considered as a benchmark production plant by extending it to simulate continuous operation, as follows:

$$\mu_X = \eta_1 \left(\mu_{\max} \left(\frac{Sg}{k_{Sg} + Sg} \right) \left(\frac{1}{1 + \frac{But}{k_{But}}} \right) \left(1 - \left(\frac{Sg}{k_{St}} \right) \right) \right) \quad (1)$$

$$r_{But} = \eta_2 \left(r_{\max But} \left(\frac{Sb}{k_{Sb} + Sb} \right) \right) \quad (2)$$

Butyric acid reaction rate:

$$r_{Sb} = \eta_3 \left(r_{\max Sb} \left(\frac{Sg}{k_{SgSb} + Sg} \right) \left(\frac{1}{1 + \left(\frac{But}{k_{But}} \right)} \right) \right) - \frac{\eta_2 \left(r_{\max But} \left(\frac{Sb}{k_{Sb} + Sb} \right) \right)}{Y_{ButSb}} \quad (3)$$

Acetone reaction rate:

$$r_{Ace} = \eta_4 \left(r_{\max Ace} \left(\frac{Act}{k_{AA} + Act} \right) \right) \quad (4)$$

Acetic acid reaction rate:

$$r_{Act} = \eta_5 \left(r_{\max Act} \left(\frac{Sg}{k_{SgAct} + Sg} \right) \left(\frac{1}{1 + \frac{Ace}{k_{iAce}}} \right) \right) - \frac{\eta_4 \left(r_{\max Ace} \left(\frac{Act}{k_{AA} + Act} \right) \right)}{Y_{AceAct}} \quad (5)$$

Ethanol reaction rate:

$$r_{Et} = \eta_6 \left(r_{\max Et} \left(\frac{Sg}{k_{SgEt} + Sg} \right) \right) \quad (6)$$

Glucose mass balance:

$$\frac{dSg}{dt} = \frac{F}{V} (Sg_a - Sg) - \left(\frac{\mu_X X}{Y_{XSg}} \right) \quad (7)$$

Biomass mass balance:

$$\frac{dX}{dt} = -\alpha \frac{F}{V} X + (\mu_X - k_{spo}) X \quad (8)$$

Butanol mass balance:

$$\frac{dBut}{dt} = -\frac{F}{V} (But) + r_{But} X \quad (9)$$

Butyric acid mass balance:

$$\frac{dSb}{dt} = \frac{F}{V} (Sb_a - Sb) + r_{Sb} X \quad (10)$$

Acetic acid mass balance:

$$\frac{dAct}{dt} = -\frac{F}{V} (Act) + r_{Act} X \quad (11)$$

Acetone mass balance:

$$\frac{dAce}{dt} = -\frac{F}{V} (Ace) + r_{Ace} X \quad (12)$$

Ethanol mass balance:

$$\frac{dEt}{dt} = -\frac{F}{V} (Et) + r_{Et} X \quad (13)$$

The kinetic model is composed of 6 kinetic rates (Eqs. (1–6)) and a set of seven differential equations (Eqs. (7–13)) describing the corresponding mass balances for Glucose (Sg), Biomass (X), Butanol (But), Butyric acid (Sb), Acetic acid (Act), Acetone (Ace) and Ethanol (Et) respectively. *F* stands for the volumetric flow of the feeding solution, *V* stands for the volume of culture medium within the reactor and *Sg_a* and *Sb_a* are the glucose and butyric acid concentrations within the feeding solution.

The analysis of the simplified metabolic pathway of *C. acetobutylicum* reported by Haus *et al.* [6], which is shown in Fig. 1, was used to propose the mathematical structures representing the reaction rates within the system. It describes that the metabolism of glucose oxidation is carried out in two sequential phases, one phase called acidogenesis, carried out by vegetative cells, which includes from the glycolysis pathway up to the formation of Acetyl CoA and its subsequent oxidation to organic acids such as butyric, lactic and acetic; and another metabolic pathway called solventogenesis, performed in conjunction with the metabolism that triggers the process of sporulation, which starts from the reincorporation of the organic acids mentioned above into the cell and culminates with its transformation into acetone and butanol [21]. In the case of ethanol, it is reported in the literature that its production is constitutive, regardless of the metabolic state of the crop. It is also important to note that the strain *C. acetobutylicum* ATCC 824 has not been genetically modified to inhibit its sporulation process, so this effect was also considered within the equation construction.

In the case of the characterization of the system operating with immobilized cells, a pseudo-homogeneous approach was considered to characterize the effect that diffusion limitations have on the system based on the application of the so-called efficiency factor (η). This parameter is defined as the quotient of the apparent reaction rate considering diffusion restrictions between the reaction rate observed in a system without limitation by mass transfer, so it can take values between 0 and 1 [22].

Finally, to consider the fact that the biomass inside the reactor in the immobilized cell system is attached to the support and this is suspended inside the reactor, it was considered to include an additional parameter in the mass balance for that variable, called α . This parameter represents the mass fraction of biomass leaving the reactor due to the input–output mass flow under continuous regime. Alfa value can range from 0 to 1, where $\alpha = 0$ represents packed-bed flow dynamics and $\alpha = 1$ represents the ideal case of the "perfect mixing" continuous reactor [23].

The parametric identification of the proposed model was made via the Marquardt algorithm into the software ModelMaker® 3.0.3. Experimental data was obtained from a fermentation system reported by Yen and Li [24], which consisted of a stirred tank reactor of 1 L nominal volume working with 600 mL of P2 medium with glucose as the main carbon source and inoculated with cells of *C. acetobutylicum* ATCC 824, operated under four different operating regimes: batch and continuous with free cells and batch and continuous with immobilized cells respectively. The immobilization medium was a powdered brick with a particle diameter between 0.15 and 2.4 mm, which occupied 65 ml of reactor volume.

2.2. Numerical simulations

All the numerical simulations were made into a PC equipped with an Intel® Core® i5 560 M processor and 4 GB of RAM into the MATLAB® 2016a software, using the ODE Solver library's command ode15s to solve the system's set of differential equations by considering the following operational regimes: batch with organic acid feeding, single stage CSTR, three stage CSTR with and without biomass recirculation and fed-batch with constant feeding rate. The theoretical performance of the system was evaluated by considering butanol titre and productivity at equilibrium state in batch culture and stationary state in all the CSTR ones.

In addition, to characterize the steady state behaviour of the systems operating under continuous regime, bifurcation diagrams were elaborated considering the dilution rate ($D = F/V$), glucose concentration in the feed stream ($S_{g,s}$) and concentration of butyric acid in the feed stream ($S_{b,s}$) as bifurcation parameters using the MATCONT version 5p0 program, which runs within the framework of MATLAB® 2016a.

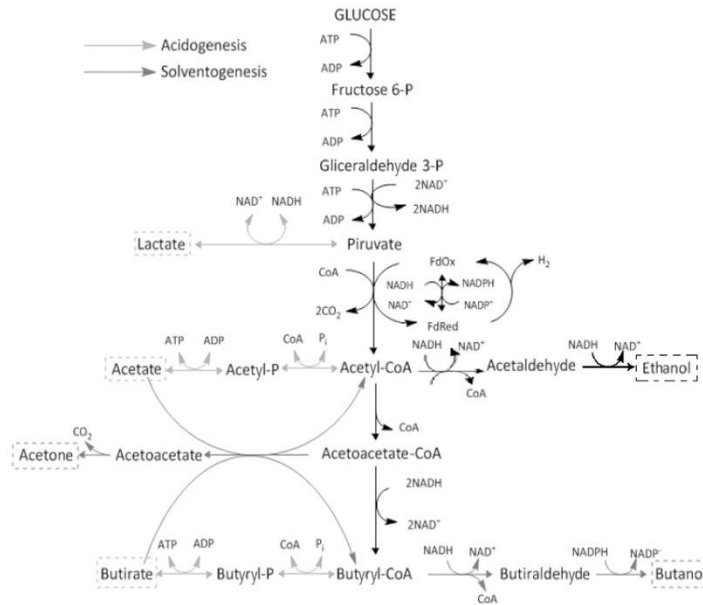


Fig. 1. Diagram of the simplified ABE fermentation pathway of *Clostridium acetobutylicum* reported by Haus *et al.* [6].

3. Results

3.1. Model validation

Table 1 summarizes the values obtained by the results of the parametric identification. The validation of the model was made by simulation considering the following initial conditions: $X_0 = 0.2 \text{ g}\cdot\text{L}^{-1}$, $S_{G0} = 60 \text{ g}\cdot\text{L}^{-1}$, $But_0 = 0.01 \text{ g}\cdot\text{L}^{-1}$, $Sb_0 = 0.01 \text{ g}\cdot\text{L}^{-1}$, $Act_0 = 0.01 \text{ g}\cdot\text{L}^{-1}$, $Et_0 = 0.01 \text{ g}\cdot\text{L}^{-1}$ (Fig. 2), where the mathematical model represents the dynamic behaviour of the analysed variables with a linear correlation index $r^2 = 0.9952$ and $r^2 = 0.9710$ over experimental data for free and immobilized cells respectively, and a p -value < 0.001 , which ensures that there is not a significant difference between the predicted behaviour described by the proposed model and the experimental data [25]. It should be noted that the parameters η_j and α only take the value reported when using the structure to model the system with immobilized cells, both in batch and continuous regime, otherwise they are assigned the value of one (Table 2).

Table 1
Previous reported mathematical models for the description of the ABE fermentation system

Refs.	Model
Papouastakis <i>et al.</i> [12]	First fermentation model used to determine product yields under batch regime via a carbon balance
Votruba <i>et al.</i> [13]	First dynamic model for batch fermentation to considerate a non-linear term for product inhibition
Honda <i>et al.</i> [14]	Dynamic model made to simulate the performance of butanol productivity in batch, fed-batch and repeated batch operation considering a constant biomass concentration after every fermentation stage
Mulchandani and Volesky [15]	Model for the description of an ABE fermentation system operating in continuous with a cell retention unit and using a discrete term to describe the inhibition by butanol based on known concentration thresholds.
Shinto <i>et al.</i> [16]	Metabolic model comprised of 19 kinetic equations representing each of the main enzymes involved into the ABE fermentation pathway
Raganati <i>et al.</i> [17]	Kinetic model for the ABE fermentation system operating in batch regime considering diverse carbon sources and their effect in the maximum growth rate of the culture.
Eom <i>et al.</i> [18]	Dynamic model applied to the description of an ex-situ butanol recovery unit via adsorption coupled to the ABE fermentation in continuous regime using a Monod-Ludeking-Piret and Extended Langmuir approach.
Thorn and King [19]	Dynamic model of the effect of pH shifts applied to a continuous culture of <i>Clostridium acetobutylicum</i> based on the representation of the acidogenic and solventogenic stages by differential inclusions.

Table 2
Parametric identification of the proposed model considering experimental data reported by Yen and Li [24]

Parameter	Value	Units	Parameter	Value	Units
K_{GA}	3.7924 ± 1.1382	$\text{g}\cdot\text{L}^{-1}$	v_{maxET}	0.0638 ± 0.044	h^{-1}
K_{SPAT}	9.8772 ± 1.1608	$\text{g}\cdot\text{L}^{-1}$	v_{maxSB}	0.7159 ± 0.0758	h^{-1}
K_{SPG}	0.1 ± 0.0590	h^{-1}	μ_{max}	0.6003 ± 0.0738	h^{-1}
K_{ACET}	16.4454 ± 6.4519	h^{-1}	$Y_{AC/ACT}$	0.4182 ± 0.1699	$\text{g}\cdot\text{g}^{-1}$
K_{SP}	3.5 ± 3.0195	$\text{g}\cdot\text{L}^{-1}$	$Y_{BU/SB}$	0.4852 ± 0.0645	$\text{g}\cdot\text{g}^{-1}$
K_{SG}	25 ± 4.9228	$\text{g}\cdot\text{L}^{-1}$	$Y_{XS/SG}$	0.1088 ± 0.0403	$\text{g}\cdot\text{g}^{-1}$
$K_{SG/ACT}$	0.5 ± 0.0684	$\text{g}\cdot\text{L}^{-1}$	α	1 or 0.26	–
$K_{SG/ET}$	0.0726 ± 0.0018	$\text{g}\cdot\text{L}^{-1}$	η_1	1 or 0.6372	–
$K_{SG/SB}$	2.51 ± 0.9926	$\text{g}\cdot\text{L}^{-1}$	η_2	1 or 0.5580	–
K_{SP}	240.5 ± 6.1425	$\text{g}\cdot\text{L}^{-1}$	η_3	1 or 0.7903	–
$Y_{maxAC/CE}$	0.6361 ± 0.1581	h^{-1}	η_4	1 or 0.6153	–
Y_{maxACT}	0.3349 ± 0.1079	h^{-1}	η_5	1 or 0.9850	–
$Y_{maxBT/ET}$	0.9949 ± 0.0684	h^{-1}	η_6	1 or 0.7130	–

The high parametric uncertainty observed in some estimated values can be attributed to the fact that experimental data is measured with finite accuracy and only a subset of the state variables is accessible experimentally in an “in-line” manner, as samples must be obtained from the culture and processed offline, this coupled with the inherent non-linearity nature of biological systems can be the main causes for such results [26]; however key parameters like the maximum specific growth rate (μ_{max}) are indeed within values reported into literature for *Clostridium* bacteria [27,17].

3.2. Batch culture with butyric acid supplementation

In a previous work, Chang [28] evaluated both theoretically and experimentally the effect of the addition of butyric acid on batch fermentation systems of *Clostridium beijerinckii*, concluding that supplementation of the culture medium using initial concentrations of butyric acid in a range between 2 and $5 \text{ g}\cdot\text{L}^{-1}$ could offer an increase of up to 20% in the butanol titre at the end of the fermentation; Chang also showed that values higher than the aforementioned range negatively impacted butanol generation since the crop could suffer a shortage of ATP, which reduced biomass growth. Fig. 3 and Fig. 4 show the behaviour of the fermentation system with both free and immobilized cells respectively, operating under batch regime with and without butyric acid supplementation. The simulation analysis done to evaluate the system response under such conditions was performed considering $F = 0 \text{ L}\cdot\text{h}^{-1}$ and $Sb_0 = 4 \text{ g}\cdot\text{L}^{-1}$. The results show that the effect of butyric acid addition in fact increases the final butanol concentration in both

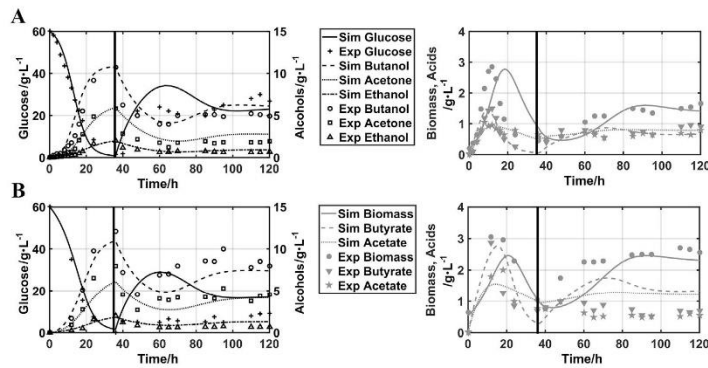


Fig. 2. Validation of the proposed kinetic model versus *Clostridium acetobutylicum* ATCC 824 experimental data reported by Yen and Li [24]: A) Free cells fermentation, $r^2 = 0.9995$ and B) Immobilized cells fermentation, $r^2 = 0.9710$. Horizontal line indicates the switch between batch and continuous regime (36 h).

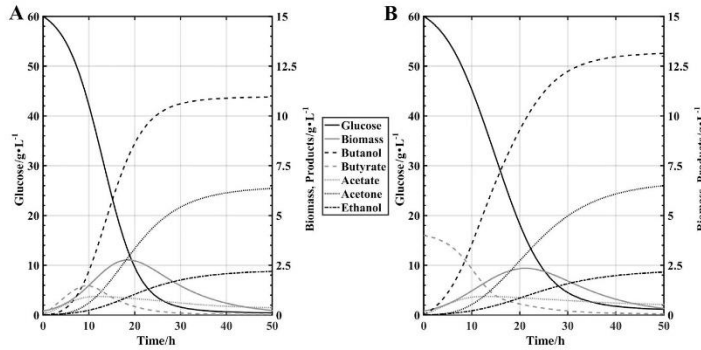


Fig. 3. Kinetic comparison of *Clostridium acetobutylicum* ATCC 824 under batch regime between: A) Free cells without butyric acid supplementation and B) Free cells with $S_{b0} = 4 \text{ g}\cdot\text{L}^{-1}$.

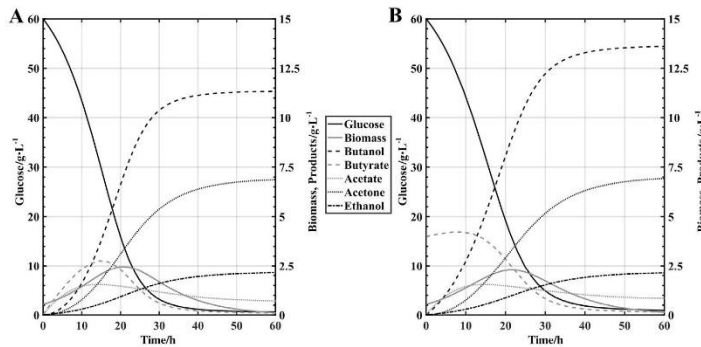


Fig. 4. Kinetic comparison of *Clostridium acetobutylicum* ATCC 824 under batch regime between: A) Immobilized cells without butyric acid supplementation and B) Immobilized cells with $S_{b0} = 4 \text{ g}\cdot\text{L}^{-1}$.

the free and immobilized cell systems by about 20%, however there is a sensitive delay of about 10 h to reach the kinetic equilibrium state in the system; This may be due to the excess production of butanol during the start of the fermentation that causes the phenomenon of inhibition by product to manifest itself in a shorter time than if there were no acid addition, so this has a negative impact on the productivity of the system. It should be mentioned that in spite of the above information, it can be corroborated that *C. acetobutylicum* ATCC 824 is also able to tolerate initial concentrations of butyric acid between 0 and $4 \text{ g}\cdot\text{L}^{-1}$ without exhibiting the phenomenon known as "acid-crash", which is the inhibition of the activation of the sporulation phase of the microorganism due to a very pronounced drop of pH below 4 units.

3.3. Fed-batch fermentation

Semi-continuous operation regime is generally used to reduce the impact of the substrate inhibition phenomenon on a culture, generally carried out by starting with a batch fermentation until either a desired substrate concentration or an equilibrium condition is reached, then a concentrated substrate solution is fed at a constant, linear, exponential or intermittent rate [29]. In ABE fermentation, fed batch culture is recommended only when there is the possibility of coupling a product recovery process in line with the fermentation due to the strong effects of butanol inhibition [30,31].

In order to study the behaviour of fed-batch culture an additional differential equation must be added to the system, as in this regime the reaction volume is not constant:

$$\frac{dV}{dt} = F \quad (14)$$

Then, a theoretical scenario was set-up considering a theoretical bioreactor of 1000 L of capacity, initiating batch operation with half of its operation volume ($V_0 = 500 \text{ L}$). As a first simulation essay, there was proposed to evaluate the behaviour of the fermentation system using an arbitrary initial $F = 5 \text{ L}\cdot\text{h}^{-1}$ and beginning the pumping of the inlet solution, containing a S_{g0} of $60 \text{ g}\cdot\text{L}^{-1}$, after 36 h of batch operation. Fig. 5 indicates that constant feeding rate of the reactor, at least with the proposed flow rate and glucose concentration, is not able to fully mitigate the effect of bacterial growth inhibition due butanol accumulation, even if its concentration in the reactor lowers from 10.95 to $7.83 \text{ g}\cdot\text{L}^{-1}$; however when the reactor volume reaches its final value of 1000 L after 136 h there is a slight recovery of bacterial growth that was able to consume the remaining glucose within the medium to produce enough butanol to reach $10.02 \text{ g}\cdot\text{L}^{-1}$.

The results of simulations condensed in Fig. 6 represent the dynamic of the fed-batch system manipulating the inlet flow F between 2 and $10 \text{ L}\cdot\text{h}^{-1}$ with a constant S_{g0} of $60 \text{ g}\cdot\text{L}^{-1}$, these conditions were chosen

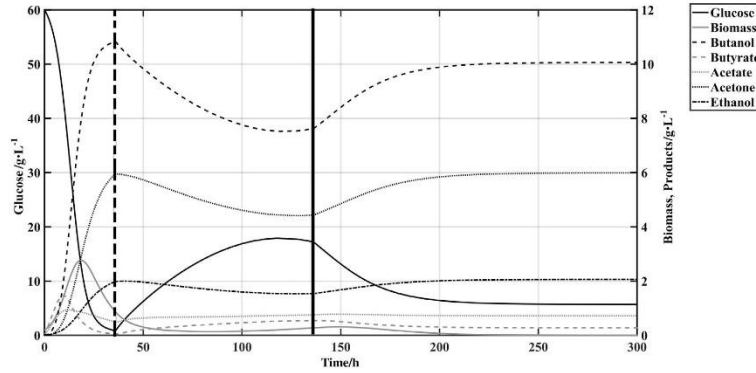


Fig. 5. Simulation results obtained with the model proposed in this work considering fed-batch operation regime with a constant feeding rate $F = 5 \text{ L} \cdot \text{h}^{-1}$ containing glucose at $60 \text{ g} \cdot \text{L}^{-1}$; the dotted vertical line indicates the beginning of the inlet flow and the solid line indicates where the flow stops due to maximum volume reached.

to avoid a drop in reaction rate by possible substrate inhibition and to ensure that the process had a minimum fermentation time of 50 h. It can be observed that for substrate feed rates less than $4 \text{ L} \cdot \text{h}^{-1}$ the amount of glucose entering the reactor is not sufficient for the crop to maintain a steady growth rate to counteract the dilution effect caused by the increase in reaction volume. However, at feed rates above $6 \text{ L} \cdot \text{h}^{-1}$ there is an increase in the production of butanol which allows to continue increasing its concentration in spite of the increase of volume, which together with the reduction in the total process time due to limitation by volume allows to increase the productivity of the system by 10%.

If the performance of the fed-batch system is measured in raw mass production then the reactor operating with immobilized cells and butyric acid supplementation at a $F = 8 \text{ g} \cdot \text{L}^{-1}$ produced 5.105 kg of butanol within the initial 36 h and after 120 h it produced 11.33 kg. Nonetheless, as butanol is traditionally recovered via distillation, the dilution effect of the product compared with the batch-only titre

(13.60 vs. $11.33 \text{ g} \cdot \text{L}^{-1}$) and also the increase in process time should be evaluated to determine the viability of this production strategy.

3.4. Single stage CSTR

For the analysis of the system operating in single-stage CSTR, the use of bifurcation analysis was used as a tool to describe the fermentation dynamics considering the dilution rate ($D = F/V$), the concentration of glucose in the stream (S_{G_s}) and the concentration of butyric acid in the feed stream (S_{b_s}) as bifurcation parameters. Within the main objective of developing bifurcation diagrams considering the aforementioned process parameters was to identify as a first step the reactor washing rate (D_w), which allowed to identify the limits of the operating region of the reactor under analysis; as well as to find out the range of values in the substrate feed concentration that can be used without promoting the inhibition of bacterial growth, this is because as reported by Qureshi *et al.* [31] *Clostridium* cells exhibit growth inhibition by glucose when its

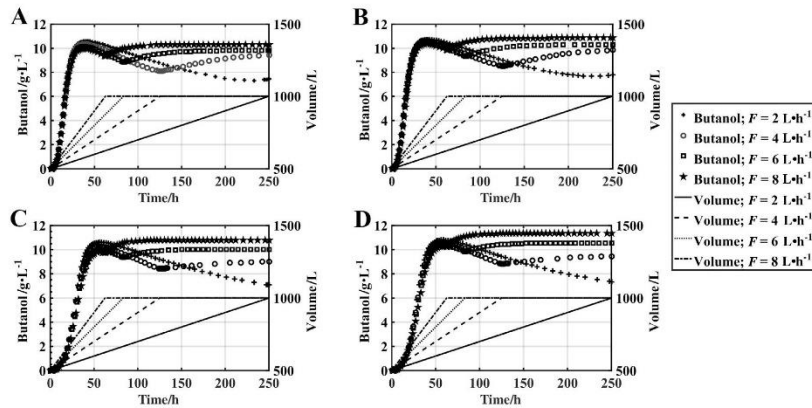


Fig. 6. Comparison between butanol production (markers) and reactor volume dynamics (lines) in cultures of *Clostridium acetobutylicum* ATCC 824 under fed-batch regime: A) free cells without butyric acid supplementation, B) free cells with $S_{b_s} = 4 \text{ g} \cdot \text{L}^{-1}$; C) immobilized cells without butyric acid supplementation and D) immobilized cells with $S_{b_s} = 4 \text{ g} \cdot \text{L}^{-1}$.

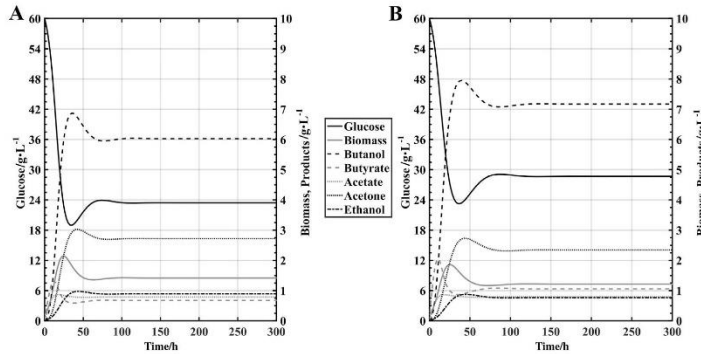


Fig. 7. Kinetic comparison of *Clostridium acetobutylicum* ATCC 824 under continuous regime between: A) Free cells without butyric acid supplementation and B) free cells with $Sb_a = 4 \text{ g}\cdot\text{L}^{-1}$.

concentration in the medium reaches values higher than $175 \text{ g}\cdot\text{L}^{-1}$. The initial conditions chosen to carry out these simulations, both for free and immobilized cells systems were those reported by Yen and Li [24]: $X_0 = 0.2 \text{ g}\cdot\text{L}^{-1}$, $Sg_0 = 60 \text{ g}\cdot\text{L}^{-1}$, $Bu_{t0} = 0.01 \text{ g}\cdot\text{L}^{-1}$, $Sb_0 = 0 \text{ g}\cdot\text{L}^{-1}$, $Et_0 = 0.01 \text{ g}\cdot\text{L}^{-1}$, $D = 0.054 \text{ h}^{-1}$ and $Sg_a = 60 \text{ g}\cdot\text{L}^{-1}$. Additionally, the supplementation of the feeding solution with a $Sb_a = 4 \text{ g}\cdot\text{L}^{-1}$ was briefly evaluated.

Simulation results made for that purpose considering the free cells reactor are shown in Fig. 7, where the reactor reached steady-state after 150 h, and it was observed that the effect of addition of butyric acid within the feeding solution improved butanol titre from 6.01 to $7.18 \text{ g}\cdot\text{L}^{-1}$, even if the biomass concentration in the reactor decreased 15%. This phenomenon is congruent with the observations made by Chang [28] and Sandoval-Espinola *et al.* [5], where it was demonstrated that a higher butyric acid pool within the medium re-routed the

metabolic carbon flow towards solvent production instead of cell formation to avoid a shortage of ATP and accumulation of NADH within the cells.

The response for the systems considering the use of immobilized biomass show similar results compared to the free cells, as the butyric acid supplementation induces an increase into butanol titre from 7.30 to $8.91 \text{ g}\cdot\text{L}^{-1}$. It should be noted that as the cells are immobilized the biomass is able to remain within the reactor up to a certain extent due to the consideration of the non-ideal solubility constant α discussed in the Model Development section, therefore the increase in both productivity and titre are higher than in the free cells scenery (19.47 vs. 22.05% respectively).

As a first step in the construction of the aforementioned bifurcation diagrams, simulations were performed to find steady state points on which the continuation algorithms of the MATCONT software could

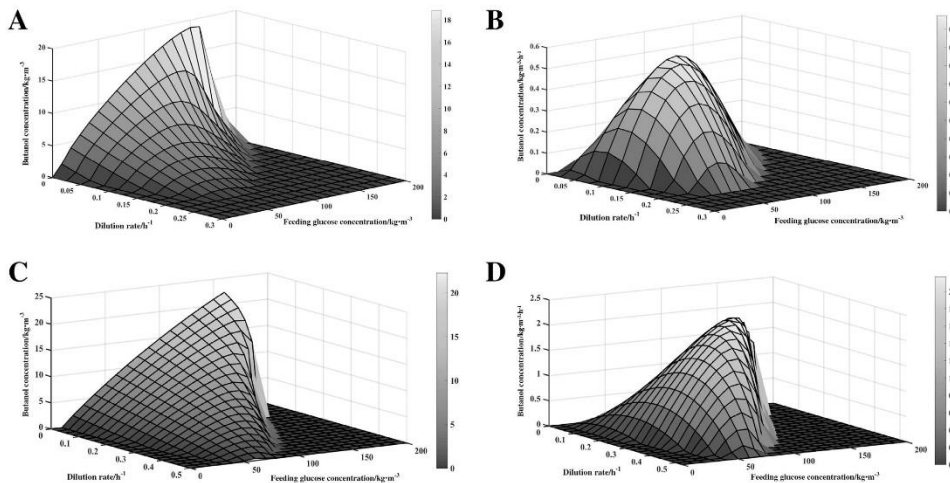


Fig. 8. Dynamics of the ABE fermentation system of *Clostridium acetobutylicum* ATCC 824 operating as CSTR with respect to changes in dilution rate (D) and glucose feed concentration (Sg_a): A) Butanol concentration in free cell system, B) Butanol productivity in the system with free cells, C) Butanol concentration in the system with immobilized cells and D) Butanol productivity in system with free cells.

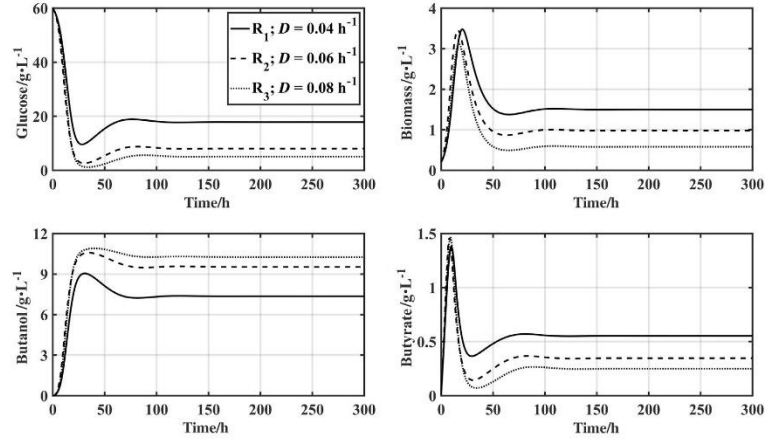


Fig. 9. Dynamic of a 3-staged CSTR system of free cells of *Clostridium acetobutylicum* ATCC 824 with biomass recirculation from the 3rd to the 1st reactor considering ascending dilution rates after every vessel.

perform the calculation of the equilibrium points reached by the fermentation system under different operational conditions. Fig. 8 shows the information regarding the bifurcation diagrams obtained for both evaluated systems (free and immobilized cells), considering as performance metrics both the steady-state butanol concentration and the butanol volumetric productivity. The large difference in concentration and productivity obtained between the reactor with free and immobilized cells is appreciated in a tangible way, this is natural considering that the proposed kinetic model includes a non-ideal flow term (α) in the biomass balance (Eq. (9)) derived from the hypothesis that the immobilization support, despite being suspended in the culture broth, does not leave the tank with the same speed as the free cells do, so that the washing effect induced by the input and output mass flow is attenuated. This same phenomenon in turn allows the reactor with immobilized cells to

operate at dilution rates of up to an order of magnitude above those that can support the system with free cells, so that its performance in both metrics evaluated is considerably higher.

Another point to note is that the proposed model has the ability to predict what glucose concentration can be exploited in the system without the occurrence of the phenomenon of inhibition by substrate ($190 \text{ g}\cdot\text{L}^{-1}$ for free cells and $160 \text{ g}\cdot\text{L}^{-1}$ for immobilized cells), while also serves to refute the theory that indicates that a higher concentration of substrate in any fermentation will always lead to a higher concentration of product. The obtained results also defy the hypothesis that a system operating with immobilized cells must be more resistant to inhibitory phenomena due to diffusional difficulties that are imposed under such conditions, nevertheless for this particular case the explanation derives from the fact that the system with immobilized cells, by

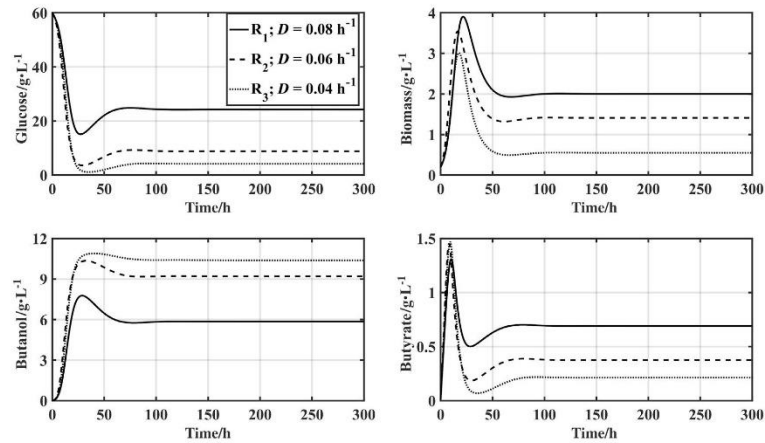


Fig. 10. Dynamic of a 3-staged CSTR system of free cells *Clostridium acetobutylicum* ATCC 824 with biomass recirculation from the 3rd to the 1st reactor considering descending dilution rates after every vessel.

producing a greater amount of butanol, is inhibited by the accumulation of this before the inhibition by glucose can be presented as such, considering that the immobilization method used in the Yen and Li [24] is based on adsorption to a porous matrix, so that as such only a portion of the immobilized cell population is subject to diffusion limitations that protect them from the action of the solvent.

In addition, in this particular operating regime it is evident that both metrics evaluated present conflicting behaviours: the conditions that allow to increase the productivity of the system force the concentration of butanol to be reduced, which is counterproductive from the point of view of the cost of downstream operations in this process, so finding operating regions that allow a balance between the two metrics could require the use of multi-objective optimization tools.

3.5. Multiple stage CSTR

According to the proposals made by de Gooijer *et al.* [32] a stepwise fermentation becomes favourable when the inhibition by product is strong, which in fact is ideal for the production of butanol; on the other hand a three-stage process is the closest to the ideal CSTR followed by a piston flow reactor. Some researchers have tried to take advantage of *Clostridium*'s two-phase metabolism (acidogenesis and solventogenesis) to propose continuous reactor operation in serial policies [33,34], considering the hypothesis that high dilution rates established in the first reactor to maximize acid production are favourable for a second stage of butanol production at a lower dilution rate, however there is strong experimental evidence which suggests that the dominant metabolism during the first culture stage becomes the control metabolism of the whole process, regardless of the dilution rate and pH set in the remaining reactors [35,36].

In this study, the simulations were carried out considering the free-cell system in a three-stage process under two different conditions, one without biomass recirculation and one with biomass recirculation, and the immobilized one just considering the scenario without biomass recirculation, mirroring a previous experimental essay by Sethaku *et al.* [37]. The selected operational regime was the one in which the dilution rate increases after each stage: $D_1 = 0.04 \text{ h}^{-1}$, $D_2 = 0.06 \text{ h}^{-1}$ and $D_3 = 0.08 \text{ h}^{-1}$ by considering a fixed $F = 10 \text{ L} \cdot \text{h}^{-1}$ but descending reactor volumes equal to $V_1 = 250 \text{ L}$, $V_2 = 167 \text{ L}$ and $V_3 = 125 \text{ L}$, with $60 \text{ g} \cdot \text{L}^{-1}$ glucose in the feed in the first stage. For this purpose an additional equation was constructed which indicates that the output biomass of the third stage is recirculated to the first reactor with the restriction that the amount of recycled biomass can only be as much as the biomass present at the outlet ($D_3 X_3$):

$$\frac{dX_1}{dt} = (-D_1 X_1) + (\mu_x - kd)X_1 + (D_3 X_3) \quad (15)$$

Simulation results in all the tested conditions show a characteristic behaviour of increase of butanol concentration after each stage, which is consistent with the behaviour of CSTR reactors in series [37], nonetheless the best performance was obtained by the configuration operating with free cells and biomass recirculation, which achieved a steady-state butanol titre of $10.25 \text{ g} \cdot \text{L}^{-1}$ (Fig. 9) versus the obtained by the immobilized cells reactor of $9.45 \text{ g} \cdot \text{L}^{-1}$. This dynamic is expected due to the fact that the system with free cells carry over more biomass at the outlet of every stage to the next one, which allows the system to maintain a higher and more uniform conversion rate through the fermentation.

The opposing dilution rate regime (descending values) was also evaluated, and as although the increase in butanol concentration between the successive reactors does indeed rise, the performance gains between stages is more pronounced. This can be explained considering that starting the fermentation with a high dilution rate cells are less prone to suffer inhibition by butanol accumulation as this component is having a relative low retention time to trigger sporulation, which can mean the successive stages get more viable cells than the

ones present within the ascending dilution rate regime (Fig. 10). The best performance obtained under such regime was achieved when there was biomass recirculation, where the final butanol titre and productivity were $10.39 \text{ g} \cdot \text{L}^{-1}$ and $103.9 \text{ g} \cdot \text{h}^{-1}$.

To complete the analysis, a comparative table of the performance of the operating regimes evaluated against the metrics obtained by the commercial fermentation system reported by Jiang *et al.* [7], where it can be seen that the use of reactors with immobilized cells can be an alternative of application to improve said industrial process (Table 3).

4. Conclusions

In this work, the performance of the ABE fermentation under different reactors configurations is analysed. For this, studio is considered as a class of unstructured phenomenological kinetic model which is able to describe the system behaviour under a greater array of operational conditions and regimes, considering only the nominal parametric identification, which sets it as an important advance towards the use of numerical simulation for the analysis and synthesis of novel butanol

Table 3
Performance comparison between an industrial ABE fermentation systems versus simulation results obtained in this work via numerical simulation under different operational regimes

Reactor configuration	Operational conditions	Butanol titre ($\text{g} \cdot \text{L}^{-1}$)	Butanol productivity ($\text{g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$)
Feed-batch [7]	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $t = 72 \text{ h}$	12.95	0.1799
Free cells batch with butyric acid	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 4 \text{ g} \cdot \text{L}^{-1}$ $t = 50 \text{ h}$	13.14	0.2628
Immobilized cells batch with butyric acid	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 4 \text{ g} \cdot \text{L}^{-1}$ $t = 50 \text{ h}$	13.60	0.2720
Free cells fed-batch with butyric acid	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 0 \text{ g} \cdot \text{L}^{-1}$ $S_{Gc} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{Bc} = 4 \text{ g} \cdot \text{L}^{-1}$ $F = 8 \text{ L} \cdot \text{h}^{-1}$ $t = 120 \text{ h}$	10.82	0.0902
Immobilized cells fed-batch with butyric acid	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 0 \text{ g} \cdot \text{L}^{-1}$ $S_{Gc} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{Bc} = 4 \text{ g} \cdot \text{L}^{-1}$ $F = 8 \text{ L} \cdot \text{h}^{-1}$ $t = 120 \text{ h}$	11.33	0.0944
Free cells continuous with butyric acid	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 0 \text{ g} \cdot \text{L}^{-1}$ $S_{Gc} = 90 \text{ g} \cdot \text{L}^{-1}$ $S_{Bc} = 4 \text{ g} \cdot \text{L}^{-1}$ $D = 0.025 \text{ h}^{-1}$ $t = 120 \text{ h}$	13.04	0.3260
Immobilized cells continuous with butyric acid	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 0 \text{ g} \cdot \text{L}^{-1}$ $S_{Gc} = 110 \text{ g} \cdot \text{L}^{-1}$ $S_{Bc} = 2 \text{ g} \cdot \text{L}^{-1}$ $D = 0.15 \text{ h}^{-1}$ $t = 120 \text{ h}$	13.08	1.9620
Free cells 3-stage CSTR with biomass recirculation	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 0 \text{ g} \cdot \text{L}^{-1}$ $S_{Gc} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{Bc} = 0 \text{ g} \cdot \text{L}^{-1}$ $D_1 = 0.08 \text{ h}^{-1}$ $D_2 = 0.06 \text{ h}^{-1}$ $D_3 = 0.04 \text{ h}^{-1}$ $t = 120 \text{ h}$	10.39	0.4156
Immobilized cells 3-stage CSTR	$S_{G0} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{B0} = 0 \text{ g} \cdot \text{L}^{-1}$ $S_{Gc} = 60 \text{ g} \cdot \text{L}^{-1}$ $S_{Bc} = 0 \text{ g} \cdot \text{L}^{-1}$ $D_1 = 0.04 \text{ h}^{-1}$ $D_2 = 0.06 \text{ h}^{-1}$ $D_3 = 0.08 \text{ h}^{-1}$ $t = 120 \text{ h}$	9.45	0.7560

S_{G0} = Initial glucose, S_{B0} = Initial butyrate, S_{Gc} = Feeding glucose concentration, S_{Bc} = Feeding butyrate concentration, t = Reaction time, D = Reactor's dilution rate.

producing bioprocesses, the simulation results obtained in this work are congruent with experimental reports.

As a notorious result it was interesting to find that the supplementation of the culture medium for ABE fermentation with butyric acid does indeed provide a slight increase in butanol concentration in both batch and fed-batch regimes, however further economic studies should be made to determine if the addition of such organic acid is viable regarding feedstock costs, as the obtained butanol concentration enhancement lies between 16% and 20%.

Additionally, it was shown that ABE fermentations operating in continuous regime and using immobilized cells have a very positive impact on the butanol titre and the productivity of the system, obtaining enhancement of both metrics accounting for an increase in 1.01% and 909% over the most recent industrial process reported to date respectively. This result is also consistent with previous experimental work made under such process conditions and can help to establish new design and process synthesis criteria to improve the viability of biological butanol production as fuel extender.

Nomenclature

D_{wi}	Reactor's washout dilution rate, h^{-1}
k_{AA}	Acetic acid-acetone affinity constant, $g \cdot L^{-1}$
k_{But}	Butanol growth inhibition constant, $g \cdot L^{-1}$
k_d	Specific cell death rate, h^{-1}
k_{SD}	Butanol-butyric acid affinity constant, $g \cdot L^{-1}$
k_{SISg}	Butyric acid-glucose affinity constant, $g \cdot L^{-1}$
k_{Sg}	Glucose affinity constant, $g \cdot L^{-1}$
k_{SgAct}	Glucose-acetic acid affinity constant, $g \cdot L^{-1}$
k_{Sgb}	Glucose-butanol affinity constant, $g \cdot L^{-1}$
k_{Sge}	Glucose-ethanol affinity constant, $g \cdot L^{-1}$
k_{SI}	Glucose growth inhibition constant, $g \cdot L^{-1}$
Y_{AceSg}	Acetone per glucose mass yield, $g \cdot g^{-1}$
Y_{ButSd}	Butanol per butyric acid mass yield, $g \cdot g^{-1}$
Y_{ButSg}	Butanol per glucose mass yield, $g \cdot g^{-1}$
Y_{EtSg}	Ethanol per glucose mass yield, $g \cdot g^{-1}$
Y_{XSG}	Biomass per glucose mass yield, $g \cdot g^{-1}$
μ_{max}	Maximum specific cell growth rate, h^{-1}
v_{maxAce}	Maximum specific acetone production rate, h^{-1}
v_{maxAct}	Maximum specific acetic acid production rate, h^{-1}
v_{maxBut}	Maximum specific butanol production rate, h^{-1}
v_{maxEt}	Maximum specific ethanol production rate, h^{-1}
v_{maxSd}	Maximum specific butyric acid production rate, h^{-1}

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Anexo 2: “Dynamic Nonlinear Feedback Control Applied to Improve Butanol Production by *Clostridium acetobutylicum*”.

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Dynamic Nonlinear Feedback Control Applied to Improve Butanol Production by *Clostridium acetobutylicum*

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Abstract:

The goal of this work is to present a closed-loop operational strategy in order to improve the butanol production in an anaerobic continuous bioreactor for the called Acetone-Butanol-Ethanol (ABE) process. The proposed control scheme considers a class of feedback signal which includes a nonlinear bounded function of the regulation error. The control scheme is applied to a phenomenological unstructured kinetic model obtained from an experimental and metabolic study of butanol production by *Clostridium acetobutylicum*, which allows the proposed structure to predict several operational conditions from batch and continuous regimes. Numerical experiments using the proposed model considering continuous operation were performed in order to find a feasible operating region for maximum butanol production at open-loop regime. The proposed methodology is applied to regulate the product concentration, manipulating the dilution rate to lead to a higher butanol productivity. The closed-loop behaviour of the bioreactor is analysed, finding that the proposed controller minimizes the response time of the system and allows it to achieve a productivity gain of 55 % over open-loop operation. Further numerical experiments show the satisfactory closed-loop performance of the proposed methodology in comparison with a PI controller.

Keywords: biofuel, butanol, clostridium, process intensification, nonlinear feedback

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1 Introduction

Acetone, butanol and ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fermentations. It was ranked second only to ethanol fermentation by yeast in its scale of production, and is one of the largest biotechnological processes ever known. Butanol is an important industrial solvent and potentially a better fuel extender than ethanol (Wallner, Ickes, and Lawyer 2012). In a typical ABE fermentation using glucose as carbon source, butyric, lactic and acetic acids by *C. acetobutylicum*, in the culture medium the pH drops and undergoes a metabolic shift, and butanol, acetone and ethanol are formed; however the butanol yield from glucose is low, typically around 15 percent w/w (Qureshi and Ezeji 2008). ABE fermentation is severely limited by product inhibition as even butanol concentrations over one percent can significantly impact cell growth and consequently the fermentation process.

Optimizing the ABE fermentation process has long been a goal of the industry. The currently developed pilot processes include cell recycling and cell immobilization reactors in order to increase cell density, productivity, and using *in situ* extractive fermentations to minimize product inhibition. However, despite the above mentioned efforts, the best results ever obtained for ABE fermentations, to date, are still less than two percent in butanol concentration, 4.46 g L⁻¹ h⁻¹ productivity, and a solvent yield of less than 25 % from glucose, and therefore had proven that they aren't efficient enough in relation to the time and effort required for their implementation (Qureshi, Saha, and Cotta 2007).

An approach to solve the above drawback is to generate novel kinetic models that could allow to design more efficient process schemes. However, as it is well known that biological reactors are typically governed

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by highly nonlinear behaviour occurring on both, macroscopic reactor scale and microscopic cellular scale, dynamic simulation is an important and useful tool for model validation, but some drawbacks can be identified; for systems with slow transient behaviour, the dynamic analysis is inefficient and potentially inconclusive. From the above it is important to find new ways to analyse such systems that could provide a more detailed overview of their performance under a wide array of operational conditions.

Bifurcation analysis is a powerful tool for evaluating time depending models applied to continuous bioreactors. The objective of bifurcation theory is to characterize changes in the qualitative dynamic behaviour of a nonlinear system as key parameters are varied, generating a picture of the model behaviour in the form of a bifurcation diagram, which can be used to determine if the model supports the steady-state and dynamic behaviour observed experimentally. When this analysis is applied considering key parameters as the bifurcation ones it's possible to predict the set of operative conditions that could lead the system to a set of high production steady states and discern which one of them is stable or not.

Additionally, a continuous bioreactor control is usually to avoid washout which could cause the reaction to stop so it can maintain a desirable production rate. This may be done by closing one feedback loop and controlling cell mass or substrate concentrations (Zhao and Skogestad 1997).

In this regard, the early successful application control strategy in process control is in the evolution of the PID controller with the traditional Ziegler-Nichols tuning method (Ziegler and Nichols 1942). Till nowadays, a high percent of the controllers implemented in the process industries are PID-type (Nikačević et al. 2012). However since the high-nonlinear behaviour of bioprocess and the adaptive mechanism of the microorganisms, is need to design novel control algorithms for improving the bioreactor yield and ensure a safety operational regime (Spear 2005; Munasinghe and Nakamura 2007; Xu and Yu 2010). Recently, alternative bounded functions have been proposed for estimation and control purposes with satisfactory performance (López-Pérez, Neria-González, and Aguilar-López 2015; Aguilar-López et al. 2016).

Therefore, in this paper a novel nonlinear controller with a class of bounded sigmoid output feedback in order to provide stabilization to a class of continuous bioreactor for butanol production is designed. The controller is aimed to regulate the butanol concentration within the reactor to the corresponding set point and thus lead the butanol productivity to higher values. The results validation was done by comparing the reactor's overall maximum theoretical productivity under open loop, versus the effect of the proposed control law at closed loop operation.

2 Methodology

2.1 Process modelling

Biochemical reactions are generally modelled by the so-called unstructured kinetic models, such structures make use of empirical mathematical structures defined as specific cell growth rates in order to describe cell growth and then, to represent the corresponding substrates consumption and products generation a pseudo-stoichiometric mass balance is used. However, this approach is generally unsuitable to predict accurately the fermentation behaviour in continuous and semi-continuous regimes, as they usually require multiple parametric sets to account for the effect of the switch of operational conditions (Eom et al. 2015; López, Passeggi, and Borzacconi 2015). In order to avoid the above mentioned drawbacks, an alternative modelling approach for biochemical systems, named phenomenological kinetic modelling, which is based on a general analysis of the metabolic pathways to propose particular kinetic models for the selected chemical species is able to predict with most accuracy the dynamic behaviour of batch, continuous and semi-continuous chemical reactors (Velázquez-Sánchez, Montes-Horcasitas, and Aguilar-López 2014). From the above, a novel phenomenological Non-Structured kinetic model was developed to describe the kinetic behaviour of ABE production in batch reactor. The mathematical model is based on classical mass balance approach and it was considered as a benchmark production plant by extending it to simulate continuous operation. The prior assumption is valid as long as the reactor can be operated near perfect mixing conditions, which can be scaled up reliability at least up to pilot level as validated experimentally for similarly modelled systems (Sforza, Enzo, and Bertucco 2014; Ariyajaroenwong et al. 2016):

Biomass reaction rate:

$$\mu_X = \left(\mu_{maxX} * \left(\frac{Sg}{kSg + Sg} \right) * \left(\frac{1}{1 + \left(\frac{But}{kBut} \right)} \right) * \left(1 - \left(\frac{Sg}{ksi} \right) \right) \right) \quad [1]$$

Butanol reaction rate:

$$r_{But} = \left(r_{maxBut} * \left(\frac{Sb}{kSb + Sb} \right) \right) \quad [2]$$

Butyrate reaction rate:

$$r_{Sb} = \left(r_{maxSb} * \left(\frac{Sg}{kSgSb + Sg} \right) * \left(\frac{1}{1 + \left(\frac{But}{kBut} \right)} \right) \right) - \frac{\left(r_{maxBut} * \left(\frac{Sb}{kSb + Sb} \right) \right)}{Y_{ButSb}} \quad [3]$$

Acetone reaction rate:

$$r_{Ace} = \left(r_{maxAce} * \left(\frac{Act}{kAA + Act} \right) \right) \quad [4]$$

Acetate reaction rate:

$$r_{Act} = \left(r_{maxAct} * \left(\frac{Sg}{kSgAct + Sg} \right) * \left(\frac{1}{1 + \left(\frac{Ace}{kIAce} \right)} \right) \right) - \frac{\left(r_{maxAce} * \left(\frac{Act}{kAA + Act} \right) \right)}{Y_{AceAct}} \quad [5]$$

Ethanol reaction rate:

$$r_{Et} = \left(r_{maxEt} * \left(\frac{Sg}{kSgEt + Sg} \right) \right) \quad [6]$$

Glucose mass balance:

$$\frac{dSg}{dt} = D(Sg_{in} - Sg) - \left(\frac{\mu_X \cdot X}{Y_{\frac{X}{Sg}}} \right) \quad [7]$$

Biomass mass balance:

$$\frac{dX}{dt} = -DX + (\mu_X - k_{spo}) * X \quad [8]$$

Butanol mass balance:

$$\frac{dBut}{dt} = -D(But) + r_{But} * X \quad [9]$$

Butyrate mass balance:

$$\frac{dSb}{dt} = -D(Sb) + r_{Sb} * X \quad [10]$$

Acetate mass balance:

$$\frac{dAct}{dt} = -D(Act) + r_{Act} * X \quad [11]$$

Acetone mass balance:

$$\frac{dAce}{dt} = -D(Ace) + r_{Ace} * X \quad [12]$$

Ethanol mass balance:

$$\frac{dEt}{dt} = -D(Et) + r_{Et} * X \quad [13]$$

The kinetic model is composed of 6 kinetic rates (1–6) and a set of seven differential eqs (7–13) describing the corresponding mass balances for Glucose (Sg), Biomass (X), Butanol (But), Butyrate (Sb), Acetate (Act), Acetone (Ace) and Ethanol (Et) respectively. The dilution rate is defined as $D = F/V$, where F stands for the volumetric flow of the feeding solution and V stands for the volume of culture medium within the reactor; and Sg_{in} is the glucose concentration within the feeding solution.

It should be noted that even if the carbon source feeding the bioreactor is obtained from agroindustrial wastes there is always the need to perform saccharification processes in order to obtain assimilable carbohydrates for bacterial growth, such as xylose, fructose, glucose, etc., where the former has been chosen for this

analysis due the wider knowledge about its catabolic pathway within the *Clostridium* cells. Therefore, the analysis of the simplified metabolic pathway of *Clostridium acetobutylicum* reported by Haus et al. (2011) which is shown in Figure 1, was used to propose the mathematical structures representing the reaction rates within the system. It describes that the metabolism of glucose oxidation is carried out in two sequential phases, one phase called acidogenesis, carried out by vegetative cells, which includes from the glycolysis pathway up to the formation of Acetyl CoA and its subsequent oxidation to organic acids such as butyric, lactic and acetic; and another metabolic pathway called solventogenesis, performed in conjunction with the metabolism that triggers the process of sporulation, which starts from the reincorporation of the organic acids mentioned above into the cell and culminates with its transformation into acetone and butanol. In the case of ethanol, it is reported in the literature that its production is constitutive, regardless of the metabolic state of the crop (Jones and Wood 1986). It is also important to note that the strain *Clostridium acetobutylicum* ATCC 824 has not been genetically modified to inhibit its sporulation process, so this effect was also considered within the equation construction.

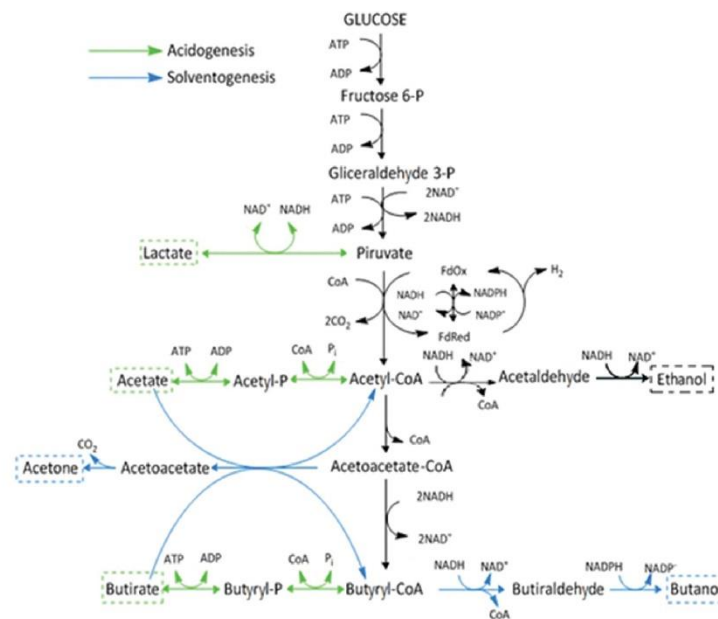


Figure 1: Diagram of the ABE metabolic pathway reported by Haus et al. (2011).

The parametric identification of the proposed model was made via the Marquardt algorithm into the software ModelMaker® 3.0.3. Experimental data was obtained from a fermentation system reported by Yen and Li (2011), which consisted of a stirred tank reactor of 1 L nominal volume working with 600 mL of P2 medium with glucose as the main carbon source and inoculated with cells of *Clostridium acetobutylicum* ATCC 824, operated under two different operating regimes: batch and continuous respectively.

2.2 Design of the proposed controller

Let us to write the system (8)–(13) in the following form:

$$\dot{z} = f(z) + \ell(z) U \tag{14}$$

Here $z \in \mathbb{R}^n_+$ is the corresponding state vector. The system (14) satisfies the Bounded Input Bounded State (BIBS) property (Basting and Dochain 1990), therefore the corresponding trajectories remain bounded. We will assume that the vector field $f(z) : \mathbb{R}^n \rightarrow \mathbb{R}^n$ is continuously differentiable in some positive invariant domain $\Omega \subset \mathbb{R}^n$ with; $f(0) = 0$; and $\sup f(z) = L$; $\forall z$, with $L < \infty$ and $\sup \ell(z) = G < \infty$.

Where:

$$z = [S_g, X, But, S_b, Act, Ace, Et] \in \Omega$$

$$f(z) = \begin{bmatrix} -\left(\frac{\mu_X X}{Y_{X/S_g}}\right) \\ (\mu_X - k_{S_{p0}}) * X \\ (r_{But} X) \\ r_{Sb} X \\ (r_{Act} X) \\ (r_{Ace} X) \\ (r_{Et} X) \end{bmatrix}$$

$$\ell(z) = \begin{bmatrix} (S_{g_{in}} - S_g) \\ -X \\ -But \\ (S_{b_{in}} - S_b) \\ -Act \\ -Ace \\ -Et \end{bmatrix}$$

$$U = D$$

Defining z_{sp} as the required trajectory, where sp means set point. Defining the control error $ase = z - z_{sp}$, from the above, the expression for the error dynamics is the following, considering a regulation control case, i. e. $z_{sp} = \text{constant}$.

$$\dot{\varepsilon} = f(z) + \ell(z) U \quad [15]$$

Proposition 1. The following control input stabilize the system (14) around a desired set point within the domain physically realizable:

$$U = k_0 g$$

$$\dot{g} = -\frac{1}{2} \left(\tanh\left(\frac{\varepsilon - a_1}{w_1}\right) - \tanh\left(\frac{\varepsilon - a_2}{w_2}\right) \right) \quad [16]$$

with $k_0, w_1, w_2 \in \mathbb{R}$.

Sketch of Proof. In order to prove the closed-loop system's stability, let us to consider the dynamic equation of the control error ε , as follows:

$$\dot{\varepsilon} = f(z) + \ell(z) k_0 g$$

$$\dot{g} = -\frac{1}{2} \left(\tanh\left(\frac{\varepsilon - a_1}{w_1}\right) - \tanh\left(\frac{\varepsilon - a_2}{w_2}\right) \right) \quad [17]$$

Now a Lyapunov based formulation for the stability analysis of the dynamics of system (15). The concept of stability in the sense of Lyapunov is closely related to that of continuity of solutions of dynamical systems. An equilibrium is stable if all solutions starting at nearby points stay nearby; otherwise, it is unstable. It is asymptotically stable if all solutions starting at nearby points not only stay nearby, but also tend to the equilibrium point as time approaches infinity.

For several classes of dynamical systems described by ordinary differential equations, the existence of the named Lyapunov functions is a necessary and sufficient condition for stability. Whereas there is no general techniques for constructing Lyapunov functions for ordinary differential equations, in several specific cases, the construction of Lyapunov functions can be known. For instance, quadratic-type functions suffice for dynamic systems related with conservation laws can often be used to construct Lyapunov functions for physical systems (Khalil 1996).

The following Lyapunov candidate function:

$$L = \varepsilon^T J \varepsilon = \varepsilon_j^2, J = J^T > 0 \quad [18]$$

Note that the proposed Lyapunov function is a standard quadratic form of the control error, where J is a positive definite symmetric matrix; therefore $L(0) = 0$ and $L(\varepsilon) > 0$ for $\varepsilon \neq 0$ as required (Bacciotti and Rosier 2005).

2.3 Numerical simulations

All the numerical simulations were made into a PC equipped with an Intel® Xeon® E5450 processor and 8 GB of RAM into the 64-bits version of the MATLAB® 2016a software, first using the ODE Solver library's command `ode15s` to solve the system's set of differential equations at an undefined time-span assuming continuous mode operation until steady state was observed, then for bifurcation analysis the system was solved employing the `Matcont 5p0` toolbox for the generation of the phase diagrams considering the reactor's dilution rate (D) and glucose feeding concentration ($S_{g,m}$) as bifurcation parameters.

3 Results and discussion

3.1 Model validation

Table 1 summarizes the values obtained by the results of the parametric identification. The validation of the kinetic model was made by numerical simulation considering the following initial conditions: $X_0 = 0.2 \text{ Kg m}^{-3}$, $S_{g0} = 60 \text{ Kg m}^{-3}$, $But_0 = 0.01 \text{ Kg m}^{-3}$, $Sb_0 = 0.01 \text{ Kg m}^{-3}$, $Acc_0 = 0.01 \text{ Kg m}^{-3}$, $Act_0 = 0.01 \text{ Kg m}^{-3}$ and $Et_0 = 0.01 \text{ Kg m}^{-3}$, and operational conditions for $D = 0.054 \text{ h}^{-1}$ and $S_{g,m} = 60 \text{ Kg m}^{-3}$ (Yen and Li 2011), where the mathematical model represents the dynamic behaviour of the analysed variables with a linear correlation index $r^2 = 0.9952$ and $r^2 = 0.9710$ over experimental data for batch and continuous culture respectively, and a p -value < 0.001 which ensures there is not a significant difference between the predicted behaviour described by the proposed model and the experimental data (Sellke, Bayarri and Berger, 2011). The results are showcased in Figure 2.

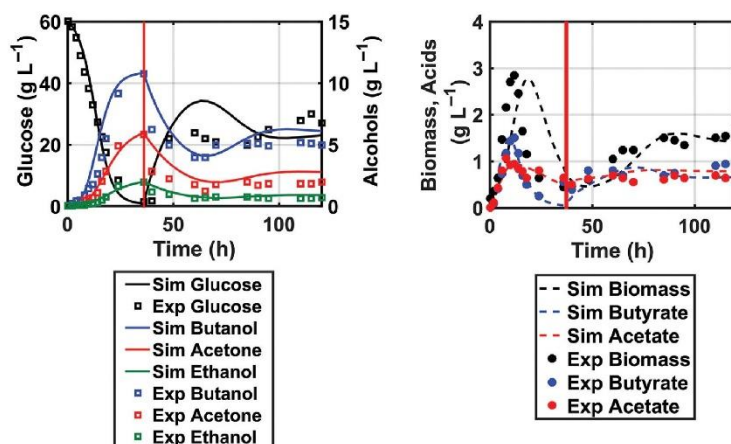


Figure 2: Validation of the proposed kinetic model versus *Clostridium acetobutylicum* ATCC 824 experimental data reported by Yen and Li (2011): Batch fermentation, $r^2 = 0.9995$ and continuous fermentation, $r^2 = 0.9710$. Horizontal line indicates the switch between batch and continuous regime (36 h).

Table 1: Parametric identification of the proposed model considering experimental data reported by Yen and Li (2011).

Parameter	Value	Units	Parameter	Value	Units
k_{AA}	4.3115 ± 13.446	Kg m^{-3}	$v_{\max,Acc}$	0.6687 ± 0.1764	h^{-1}
k_{But}	8.5 ± 2.7447	Kg m^{-3}	$v_{\max,Act}$	0.4716 ± 0.1078	h^{-1}
k_{Spo}	0.099 ± 0.0615	h^{-1}	$v_{\max,But}$	0.9949 ± 0.3656	h^{-1}
$k_{i,Acc}$	25 ± 2.9482	h^{-1}	$v_{\max,Et}$	0.0340 ± 0.0025	h^{-1}
$k_{i,Sb}$	2.25 ± 1.2741	Kg m^{-3}	$v_{\max,Sb}$	0.9949 ± 0.1162	h^{-1}
k_{Sg}	24.9999 ± 2.9178	Kg m^{-3}	μ_{\max}	0.6 ± 0.1211	h^{-1}

k_{SgAct}	0.5942 ± 0.0542	Kg m^{-3}	Y_{AceAct}	0.2681 ± 0.0775	Kg Kg^{-1}
K_{SgEt}	0.0535 ± 0.0045	Kg m^{-3}	Y_{ButSb}	0.6 ± 0.0941	Kg Kg^{-1}
k_{SgSb}	9.9962 ± 3.2172	Kg m^{-3}	Y_{XSg}	0.1107 ± 0.0417	Kg Kg^{-1}
k_{ai}	250.9999 ± 17.7785	Kg m^{-3}			

The high parametric uncertainty observed in some estimated values can be attributed to the fact that experimental data is measured with finite accuracy and only a subset of the state variables is accessible experimentally in an *in-line* manner, as samples must be obtained from the culture and processed *off-line*, this coupled with the inherent non-linearity nature of biological systems can be the main causes for such results (Vanlier et al. 2013); however key parameters like the maximum specific growth rate (μ_{max}) are indeed within values reported into literature for *Clostridium* bacteria growing using glucose as main carbon source (Procentese et al. 2014; Raganati et al. 2015).

To further reinforce the validation of the proposed kinetic model for its use as an adequate basis for the analysis of ABE fermentation additional numerical simulations were made to compare its performance against experimental values reported by Yen and Li (2011) considering a bioreactor operating in continuous culture under different feeding glucose concentrations (Table 2) and also varying the reactor's dilution rate (Table 3), where it should be noted that the model is able to reproduce the phenomenological behaviour of the culture for butanol production, total acid production and total solvent production.

Table 2: Performance comparison of the proposed kinetic model against experimental data reported by Yen and Li (2011) for butanol, total acids and total solvent production under continuous regime at different feeding glucose concentrations at $D = 0.027 \text{ h}^{-1}$.

	Feeding glucose concentration		80 Kg m^{-3}	
	60 Kg m^{-3}			
	Yen and Li (2011)	This work	Yen and Li (2011)	This work
Total ABEs	17.25	13.54	17.44	18.58
Butanol	8.94	7.68	9.94	10.63
Total Acids	1.07	2.50	1.63	2.46

Table 3: Performance comparison of the proposed kinetic model against experimental data reported by Yen and Li (2011) for butanol, total acids and total solvent production under continuous regime at different dilution rates at $S_{g_a} = 60 \text{ Kg m}^{-3}$.

	Dilution rate		$D = 0.107 \text{ h}^{-1}$	
	$D = 0.054 \text{ h}^{-1}$			
	Yen and Li (2011)	This work	Yen and Li (2011)	This work
Total ABEs	12.9	12.79	11.28	11.34
Butanol	8.07	7.33	6.82	6.62
Total Acids	1.35	2.57	1.72	2.71

It is noteworthy that the proposed kinetic model attained an acceptable reproduction performance of the fermentation system using only the parameter set reported at the beginning of this section, which showcases that the mathematical representation of the metabolic pathway is more than adequate to cope with changes in the operational conditions of the bioreactor, however as industrial processes are moving towards the use of agroindustrial waste as raw material for ABE fermentation systems there is also the need to ensure that the structure could remain as a viable choice under such scenarios. To assess such issue Jiang et al. (2014) and Raganati et al. (2015) reported that *Clostridium* cultures are indeed affected by changes in the utilised carbon source, but the main effect was over butanol yield and fermentation time caused by a different affinity for every substrate different to glucose which mostly impacted just into its observed μ_{max} , which suggests that the proposed structure can be adapted to function considering xylose, sucrose or sugarcane juice as substrates by just the re-identification of the kinetic parameters.

3.2 Bifurcation analysis

As a first step in the construction of the aforementioned bifurcation diagrams, numerical simulations were performed to find steady state points on which the continuation algorithms of the MATCONT software could perform the calculation of the equilibrium points reached by the fermentation system under different operational conditions. The final concentrations of the modelled variables by extending the simulation time under continuous regime until steady state was reached, considering Yen and Li's (2011) reported operational conditions and experimental data. The results for said simulation were 23.4361, 1.4188, 6.0305, 0.6742, 2.7217 and 0.8916 Kg m^{-3} for residual sugar, biomass, butanol, acetate, acetone and ethanol respectively after 200 h.

With the results obtained with the prior simulations there was applied a bifurcation analysis over the dilution rate to identify operational range values for the controller to avoid washout into the reactor and also to find the theoretically maximum glucose feeding concentration ($S_{g_{in}}$) that the biological system could handle without compromising its performance due product accumulation or low substrate uptake efficiency. In this experiment the aim was to determine the final concentration of the desired metabolites at steady state and to identify the reactor's washout rate (D_W), the maximum butanol concentration and maximum butanol productivity of the fermentation system (Figure 3). From the results the identified D_W was equal to 0.2251 h^{-1} , where it is noteworthy that under the experimental conditions reported by Yen and Li (2011) the maximum dilution rate evaluated was equal to 0.107 h^{-1} and under said scenario the products concentration at steady state compared with the ones obtained at 0.054 h^{-1} were 16 % lower on average, indicating that the result obtained by simulation for the determined D_W can be considered as accurate. Additionally it must be noted that even if the correlation between $S_{g_{in}}$ and residual glucose within the reactor is always directly proportional, the behaviour of the butanol titer against the dilution rate is not. There is a region which can be identified in the diagram showed in Figure 3 that suggest there is a local maximum for butanol productivity approximately equal to $0.5 \text{ Kg m}^{-3} \text{ h}^{-1}$, with a final butanol concentration of 7.15 Kg m^{-3} , at a $D = 0.075 \text{ h}^{-1}$ and $S_{g_{in}}$ values between 110 and 120 Kg m^{-3} but not higher. Said behaviour is consistent with the fact that *Clostridium* growth rate is not only inhibited by the accumulation of the produced organic solvents, but also by substrate feeding concentrations over 150 Kg m^{-3} (Lee et al. 2008).

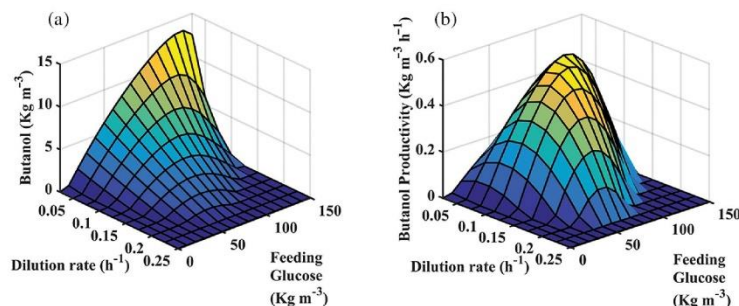


Figure 3: Dynamics of the ABE fermentation system of *Clostridium acetobutylicum* ATCC 824 operating as CSTR with respect to changes in dilution rate (D) and glucose feed concentration ($S_{g_{in}}$): (a) Butanol concentration and (b) Butanol productivity.

Therefore, as the main objective of this paper is to improve the system's butanol productivity but taking into account that the majority of its process costs are due the recovery stage, it's crucial for the implementation of closed-loop production schemes outside laboratory scale that butanol concentration could lie between 8 and 19 Kg m^{-3} , as Khöler et al. (2015) report that purification costs by distillation are inversely proportional of each other within such region.

3.3 Proposed controller performance

Table 4 indicates the obtained values for the proposed controller tuning, using the considerations mentioned into (14) and (26). For determining the controller's set-point, which corresponds to the butanol concentration into the reactor, it was resorted to using parallel simulations at different proposed values within the range between 9 Kg m^{-3} to 10 Kg m^{-3} , considering that the bifurcation analysis showed that within such product titters the productivity of the system would maintain at least 85 % of the maximum productivity reported in

Section 3.2 while keeping the butanol concentration in the outlet as high as possible within the operational region of the reactor. The selection criterion was based on the characterization of the control effort, determined as the fluctuation in the control input (D) with respect to process time and the steady state concentration of the products of interest. The value established based on the analysis described above corresponded to a value of 9.5 Kg m^{-3} , as it generates the greatest product concentration while resorting to the least control effort.

Table 4: Proposed controller tuning values obtained by the theoretical considerations made into (14) and (26).

Parameter	Value	Units
k_D	0.00275	—
a_1	0	Kg m^{-3}
a_2	0	Kg m^{-3}
w_1	1	$\text{m}^3 \text{Kg}^{-1} \text{h}^{-1}$
w_2	2	$\text{m}^3 \text{Kg}^{-1} \text{h}^{-1}$

For the performance analysis versus traditional control structures, a kinetic disturbance in the open-loop system via a 10 % increment to the dilution rate was made in order to tune the parameters of a PI controller, which would be used as a standard reference for comparison. The tuning parameters were obtained by the Internal Model Control (IMC) criteria reported by (Rivera, Morari, and Skogestad 1986) and are shown in Table 5.

Table 5: PID controller tuning values obtained by the empirical method described by Rivera, Morari, and Skogestad (1986).

Parameter	Value	Units
ΔU	0.054	h^{-1}
K_p	0.0712	—
t_i	280.5	h

Figure 4 shows the proposed controller stabilises the butanol concentration into the proposed set-point in approximately 400 h, versus the PI controller that requires more than 1000 h to attain such condition, as even if the proposed structure causes a higher overshoot of the controlled variable it also does converge faster. Furthermore an integral time absolute error (ITAE) analysis (Smith and Corripio 1985) was performed to compare the proposed structure behaviour with the application of the PI structure and tracking of the ITAE versus time is shown in Figure 5. Once again, it's seen that the proposed structure attains lower ITAE values than the PI one by more than an order of magnitude and such behaviour is maintained throughout the temporal spectrum evaluated in this essay (1000 h).

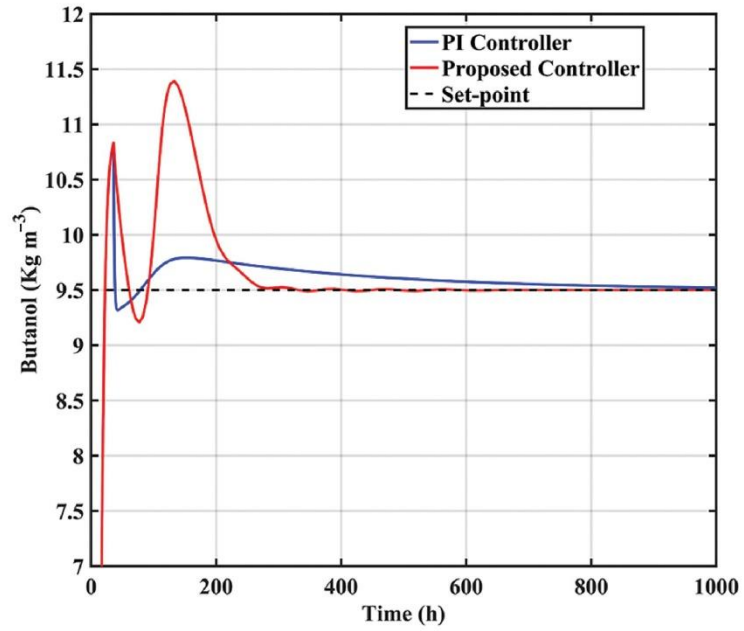


Figure 4: Comparison of the butanol concentration (controlled variable) dynamic under closed-loop operation between the proposed controller and a PI one.

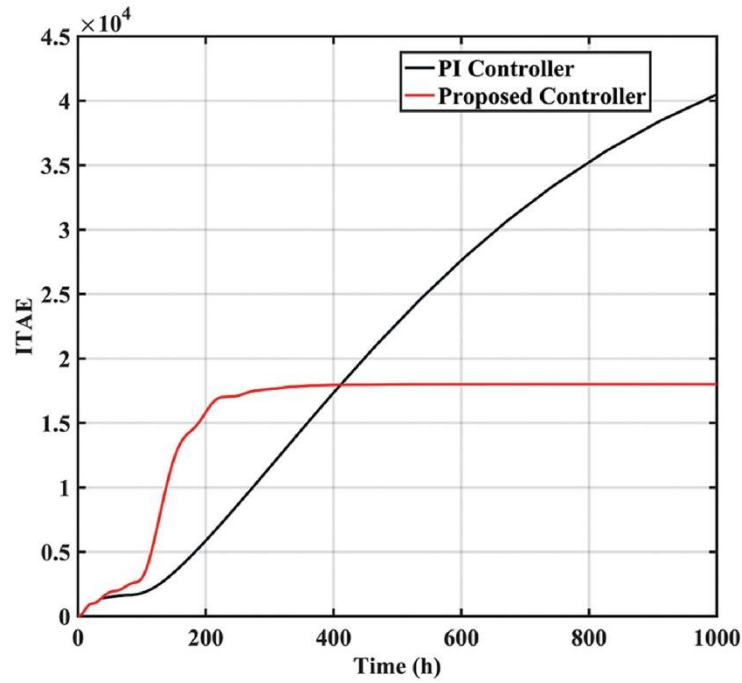


Figure 5: Evolution of the Integral Time Absolute Error performance index over time of both the proposed feedback structure and a PI controller.

In order to evaluate the system's performance, monitoring of the control effort was made, which consisted in the tracking of the dynamics of both the non-controlled variables (Figure 6) and of the dilution rate over time (Figure 7). In both cases it's shown that the control effort does indeed stabilize without visible delay after the set-point was reached and that the remaining variables do exhibit a stable behaviour, which could be inferred due the absence of critical points predicted by the model into the bifurcation analysis stage.

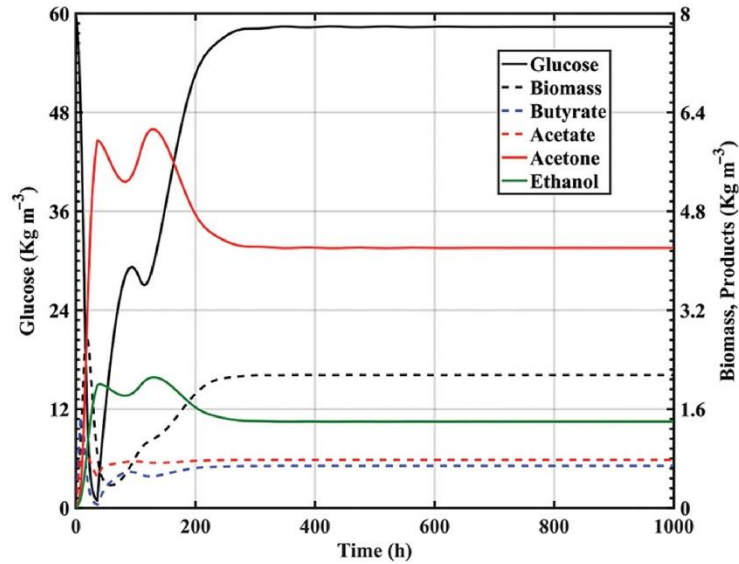


Figure 6: Dynamic of the non-controlled variables of the system under closed-loop operational regime using the proposed controller.

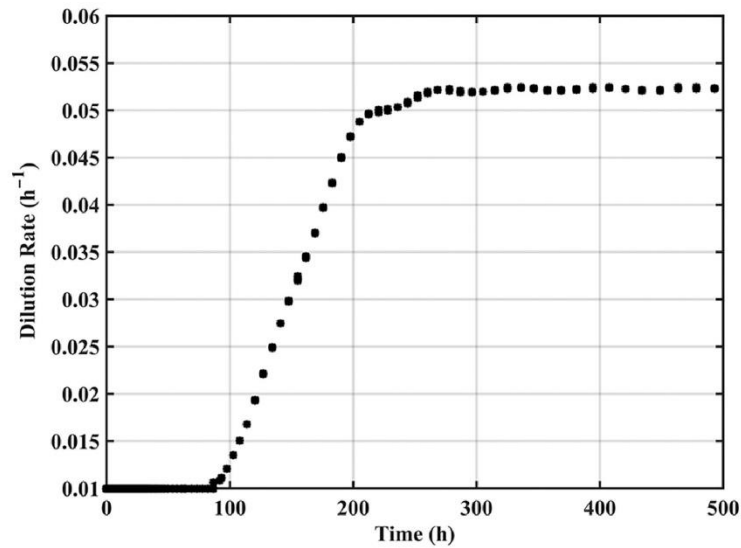


Figure 7: Dynamic of the control effort (D) of the butanol production system under closed-loop operational regime using the proposed controller.

Finally, further simulations made considering the closed-loop system demonstrated this operational regime provided both the best butanol concentration (Figure 8) and productivity results compared with the ones obtained by the open-loop simulation (Figure 9).

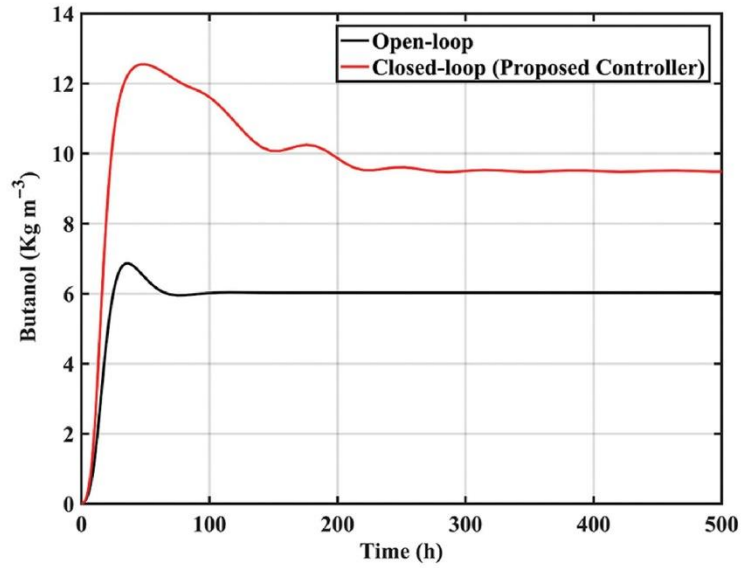


Figure 8: Comparison of the dynamic of butanol concentration between the system under open-loop and closed-loop operation.

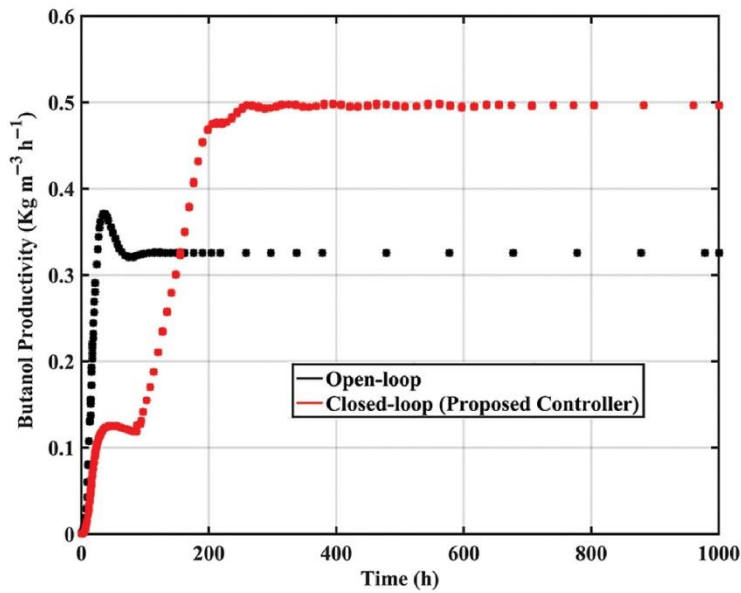


Figure 9: Comparison of the dynamic of butanol productivity between the system under open-loop (continuous line) and closed-loop (discontinuous line) operation.

As a last remark to validate the viability of the obtained results Table 6 compares the performance of the proposed closed-loop system of this work versus experimental and industrial results published into recent literature.

Table 6: Performance comparison considering butanol concentration and productivity between previously reported experimental data and this work.

Reactor configuration	Butanol concentration (Kg m ⁻³)	Butanol productivity (Kg m ⁻³ h ⁻¹)	Reference
Open-loop Industrial Fed-Batch Reactor	12.95	0.1799	Jiang et al. (2015)
Open-loop CSTR	6.01	0.3245	Yen and Li (2011)
Closed-loop CSTR	9.50	0.4995	This work

4 Conclusions

The design of a sigmoid controller is proposed to improve the butanol production in a class of anaerobic continuous bioreactor, to boost its productivity up to 56 % at high substrate feeding conditions. The proposed controller provides robustness against external disturbances, and ensure thereby conferring it a stable behaviour for long periods of time. By numerical experiments it shown that the proposed feedback structure provides a better performance compared to classical PI control. Also the controller allows the system to converge at a stable operating point with high productivity in a low settling time.

Nomenclature

k_0	Proposed controller's gain
K_{AA}	acetate-acetone affinity constant, Kg m ⁻³
K_{BA}	butyrate-acetone affinity constant, Kg m ⁻³
K_{But}	butanol growth inhibition constant, Kg m ⁻³
K_d	specific cell death rate, h ⁻¹
K_{SA}	acetate-glucose affinity constant, Kg m ⁻³
K_{Sb}	butanol-butyrate affinity constant, Kg m ⁻³
K_{SbSg}	butyrate-glucose affinity constant, Kg m ⁻³
K_{SE}	ethanol-glucose affinity constant, Kg m ⁻³
K_{Sg}	glucose affinity constant, Kg m ⁻³
K_{SgBut}	butanol-glucose affinity constant, Kg m ⁻³
r_{maxAce}	maximum acetone specific production rate, h ⁻¹ .
r_{maxAct}	maximum acetate specific production rate, h ⁻¹ .
r_{maxBut}	maximum butanol specific production rate, h ⁻¹ .
r_{maxEt}	maximum ethanol specific production rate, h ⁻¹ .
r_{maxSb}	maximum butyrate specific production rate, h ⁻¹ .
$Y_{Ace/Sg}$	acetone per glucose mass yield, Kg Kg ⁻¹
$Y_{But/Sb}$	butanol per butyrate mass yield, Kg Kg ⁻¹
$Y_{Et/Sg}$	ethanol per glucose mass yield, Kg Kg ⁻¹
$Y_{Ace/X}$	acetone per biomass yield, Kg Kg ⁻¹
$Y_{Act/X}$	acetate per biomass yield, Kg Kg ⁻¹
$Y_{But/X}$	butanol per biomass yield, Kg Kg ⁻¹

- $Y_{But/Sg}$ butanol per glucose mass yield, Kg Kg⁻¹
 $Y_{Et/X}$ ethanol per biomass yield, Kg Kg⁻¹
 $Y_{Sb/X}$ butyrate per biomass yield, Kg Kg⁻¹
 $Y_{X/Sg}$ biomass per glucose mass yield, Kg Kg⁻¹
 Greek symbols
 μ_{maxX} maximum specific cell growth rate h⁻¹

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Anexo 3: “Modelling of the pH dynamic and its effect over the Isopropanol-Butanol-Ethanol fermentation by *Clostridium acetobutylicum* pIPA3-Cm2”.

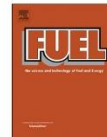
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Full Length Article

Modelling of the pH dynamic and its effect over the Isopropanol-Butanol-Ethanol fermentation by *Clostridium acetobutylicum* pIPA3-Cm2



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ABSTRACT

This work makes use of the so-called phenomenological non-structured modelling approach to propose a novel mathematical structure for the description and prediction of the pH effect over an Isopropanol-Butanol-Ethanol (IBE) fermentation system by *Clostridium acetobutylicum* pIPA3-Cm2, which includes both an in-line approximation of the pH of the culture medium and also novel expressions to reflect its real time effect over the biomass growth and metabolic response in an attempt to generate a base for the development of process intensification strategies for said fermentation system for biofuel production purposes.

The proposed model attained a correlation index $R^2 = 0.9931$ and a p -value < 0.001 versus experimental data reported by Lee et al. (2012), predicting a total of $20.1617 \text{ kg m}^{-3}$ of IBEs with a yield of $0.3341 \text{ kg}_{\text{IBE}} \text{ kg}_{\text{SS}}^{-1}$ after 42 h of batch fermentation. Additionally, both the proposed parameters for critical and optimal pH ($pH_c = 3.4977$; $pH_{op} = 6.5$) and simulation results based on the equation proposed for the modelling of the pH dynamic were consistent with experimental reports for both ABE and IBE fermentation systems under a wide array of operational conditions.

1. Introduction

Biofuels, which are all compounds of organic nature derived from living beings and their metabolism that can potentially be utilized as fuels, lead a group of alternative energy sources aimed to provide solution to the environmental issues caused by the overexploitation of fossil ones [18]. One of the most studied biofuels in the last decade is butanol, considering that it can offer better properties regarding fuel mileage yield, lower gaseous emissions, higher energy content and lower hygroscopicity versus the currently developed processes for the production of ethanol and biodiesel [6].

Traditionally, the methodology to obtain butanol via fermentation is based on the transformation of various sugars such as glucose or sucrose by Gram-positive bacteria of the genus *Clostridium*, via a metabolic pathway called ABE (acetone-butanol-ethanol) [12]. ABE fermentation presents inherent restrictions that have prevented its consolidation as a mature technology such as low production yields, inhibitory effect over the culture's growth due both solvents and glucose accumulation and mechanisms of metabolic regulation own of Gram-positive bacilli such as the sporulation process [19]. Additionally, there exists the limitation imposed by the outlet stream composition, where the low concentration of the main product (less than 3% w/v)

combined with the presence of acetone, which is deemed as undesirable if the main goal of the fermentation is to produce a biofuel capable blend, make the design and operation of *downstream* processes either too energetic demanding (via distillation) or expensive to implement (via membrane technologies) [28].

From the process engineering standpoint, in the last five years there had been advances regarding the design and implementation of alternative recovery processes such as the use of gas stripping [25], adsorption either outside the fermenter [8] or *in situ* [26] or via pervaporation [27]. And while those recovery procedures do indeed improve butanol concentration into the fermenter outlet up to 5.5 times compared with traditional batch processes those technologies must still deal with the presence of acetone in the mixture in various degrees.

As a response for such persisting inconvenient novel genetic manipulation strategies had been developed to either reduce significantly the acetone production of the Clostridial cultures [11] or transform it into a more suitable molecule for fuel purposes [21]. In this regard, Isopropanol-Butanol-Ethanol fermentation is a novel bioprocess derived from the genetic manipulation of the classic ABE-producing bacteria *C. acetobutylicum* by the introduction of the *adh_{B-523}* alcohol dehydrogenase from *C. beijerinckii*, which transforms acetone from the culture broth into isopropanol. This strategy was developed because it

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Nomenclature			
D	reactor dilution rate, h^{-1}	pH_{op}	optimal growth pH, –
k_{but}	butanol growth inhibition constant, g L^{-1}	S_{G_0}	glucose concentration in the feed stream, g L^{-1}
k_{isoA}	isopropanol-acetic acid affinity constant, g L^{-1}	Y_{butSb}	butanol per butyric acid mass yield, g g^{-1}
k_{Sb}	butanol-butyric acid affinity constant, g L^{-1}	Y_{isoAct}	isopropanol per acetic acid mass yield, g g^{-1}
k_{Sg}	glucose affinity constant, g L^{-1}	$Y_{X/Sg}$	biomass per glucose mass yield, g g^{-1}
k_{SgAct}	glucose-acetic acid affinity constant, g L^{-1}	Greek symbols	
k_{SgEt}	glucose-ethanol affinity constant, g L^{-1}	ν_{maxAct}	maximum specific acetic acid production rate, h^{-1}
k_{SgSb}	butyric acid-glucose affinity constant, g L^{-1}	ν_{maxBut}	maximum specific butanol production rate, h^{-1}
k_{Si}	glucose growth inhibition constant, g L^{-1}	ν_{maxEt}	maximum specific ethanol production rate, h^{-1}
pH_{Act}	acidity adjustment constant, –	ν_{maxIso}	maximum specific isopropanol production rate, h^{-1}
pH_c	critical pH for solventogenesis, –	ν_{maxSb}	maximum specific butyric acid production rate, h^{-1}
		μ_{maxX}	maximum specific cell growth rate, h^{-1}

docs increase the viability of the alcohol mixture as biofuel or fuel extender by removing the need to recover and segregate acetone from the reactor outlet, reducing the theoretical cost of downstream operations [10].

The implementation of fermentation technologies based on this novel IBE process requires the analysis and establishment of the proper operating conditions for the culture to express its maximum production potential to take advantage of the facilitated recovery operations. One of the most common strategies involves the selection of the adequate carbon source to maximise solvent production and growth rate of the culture [2].

Nonetheless, *Clostridium* metabolism is not only regulated at macroscopic scale only by the selection of the available carbon source, but also by the pH dynamic of its surroundings. *Clostridium* genus bacteria present at least two growth stages perfectly characterised under its life cycle. First there is the so-called acidogenic stage, where the cells partially oxidize sugar-based carbon sources to grow and to generate organic acids including butyric, acetic and lactic ones, which produce a drop on the pH medium, which after a certain threshold induces the switch to the so-called solventogenic stage, where the produced acids are reincorporated by the cells to initiate the sporulation process and then transformed into butanol and acetone in wild type strains [31].

Currently, the study of the effect of the pH dynamic into the culture medium in *Clostridium* based fermentations is primarily made via an experimental approach existing a plethora of reports into literature [9,20,1,24], where either the culture is growth under different initial

pH conditions and there is a monitoring of the resulting solvent production and growth behaviour or there are attempts to manipulate the pH of the culture towards an “optimal” pattern that could maximise the system performance via the implementation of empirical closed-loop systems at varying pH set-points and starting time politics.

Despite these experimental strategies had helped to create a framework to understand and propose operational politics for the manipulation of the pH into IBE fermentation processes there is the downside that some results are not fully reproducible or applicable due to differences over strains, reactor configurations or medium compositions across systems, which in turn would make necessary to repeat essays to readjust the conditions to match the requirements of the new system.

To try to solve such issue, mathematical modelling and numerical simulation techniques can be used to reduce the need to conduct experimental work and then serve as a basis for the application of more consistent and objective optimization and process intensification methodologies that could lead to the development of novel fermentation processes with better performance than the current established ones.

Currently there exist little previous evidence into literature of the inclusion of pH related terms for the description of metabolism changes in *Clostridium* cultures. The most recent advance in the matter are a series of works reported by Thorn, King and Sabari [22] and Millat et al. [13], where they proposed a sigmoid-like function to act over key enzymatic kinetic rates in an attempt to simulate the switch from

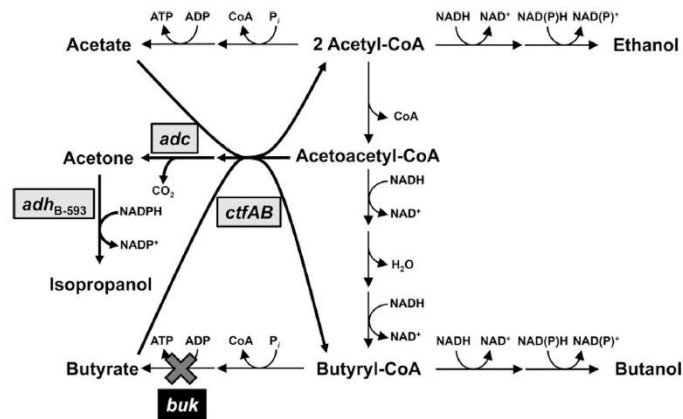


Fig. 1. Simplified diagram of the modified IBE metabolic pathway of *Clostridium acetobutylicum* pIPA3-Cm2 reported by Lee et al. [10].

acidogenesis to solventogenesis. Nonetheless, their proposals are valid only under continuous regime, which is not a viable operational regime for IBE production at industrial scale [5] and also their approaches to the ABE metabolic pathway incur in the use of a high number of state equations and parameters, and such property makes them unsuitable for its further use into process intensification methodologies.

Therefore, this work makes use of the so-called phenomenological non-structured modelling approach to propose a novel mathematical structure which includes both an in-line approximation of the pH of the culture medium and also its real time effect over the biomass growth and metabolic response in an attempt to generate a base for the development of process intensification strategies for the IBE fermentation system.

2. Methodology

2.1. Assumptions

For the generation of the kinetic model state equations, the modified *Clostridium acetobutylicum* pIPA3-Cm2 IBE metabolic pathway reported by Lee et al. [10] was used as a guideline to propose the dependences and outcomes of the kinetic rate equations (Fig. 1).

The five main states that make up the structure of the model are glucose as the limiting carbon source (Sg), biomass (X), butanol (But), isopropanol (Iso) and ethanol (Et), however it was also important to include the formation and consumption rates of both the acetic (Act) and butyric (Sb) acids, as those states would help to set the differentiation between the acidogenic and solventogenic growth stages without explicitly requiring to segregate the model equations over both stages.

In the case of ethanol, it is reported in the literature [4] that its production is constitutive, regardless of the metabolic state of the crop, which allows to represent its dynamic with a simple specific production rate only dependant on the glucose concentration within the culture medium (Eq. (1)).

$$\nu_{Et} = \left(\nu_{MaxEt} X \left(\frac{Sg}{k_{SgEt} + Sg} \right) \right) \quad (1)$$

The acidogenic stage comprises from the glucose uptake up to the biosynthetic routes of both acetic and butyric acids. Therefore it was assumed that the kinetic equations for said metabolites were only dependent on the cellular growth rate and glucose consumption (Eqs. (2) and (3)).

$$\nu_{Act} = \left(\nu_{MaxAct} X \left(\frac{Sg}{k_{SgAct} + Sg} \right) \right) \quad (2)$$

$$\nu_{Sb} = \left(\nu_{MaxSb} X \left(\frac{Sg}{k_{SgSb} + Sg} \right) \right) \quad (3)$$

For the solventogenic stage it's known that both isopropanol and butanol concentrations depend on the uptake of acetic and butyric acids respectively (Eqs. (4) and (5)), so the kinetic equations for both alcohols were proposed to be dependent on cell growth and acetate or butyrate consumption as the case may be. Nonetheless, as the solventogenic stage is triggered by a pH reduction and that there is the possibility for the system to incur into the phenomenon of acid-crash, which is the incapability of the culture to access solventogenesis due to an insufficient pH gradient between the cell outer membrane and the culture medium, then both kinetic equations were made dependent on the medium pH via a bell-like function and the addition of a controlling parameter called pH_c , which was meant to trigger the generation of solvents only if the pH into the medium is low enough (Eqs. (6) and (7)).

$$\nu_{UAct} = - \left(\frac{\nu_{MaxIso} X \left(\frac{Act}{k_{IsoA} + Act} \right)}{Y_{IsoAct}} \right) \quad (4)$$

$$\nu_{USb} = - \left(\frac{\nu_{MaxBut} X \left(\frac{Sb}{k_{Sb} + Sb} \right)}{Y_{ButSb}} \right) \quad (5)$$

$$\nu_{Iso} = \left(\nu_{MaxIso} X \left(\frac{Act}{k_{IsoA} + Act} \right) \right) \left(\frac{e^{\left(\frac{pH - pH_c}{1.5} \right)}}{\left(1.5 \left(1 + e^{\left(\frac{pH - pH_c}{1.5} \right)} \right) \right)^2} \right) \quad (6)$$

$$\nu_{But} = \left(\nu_{MaxBut} X \left(\frac{Sb}{k_{Sb} + Sb} \right) \right) \left(\frac{e^{\left(\frac{pH - pH_c}{1.5} \right)}}{\left(1.5 \left(1 + e^{\left(\frac{pH - pH_c}{1.5} \right)} \right) \right)^2} \right) \quad (7)$$

For biomass growth, it was considered that it only depends on glucose consumption and that carbon source represented the only limiting substrate, this is due the anaerobic nature of the microorganism. On the other hand, it's known that the IBE fermentation process is characterized by presenting growth inhibition both by product accumulation and high substrate concentrations [3], so the proposed growth rate includes an inhibition term for each mentioned situation (Eq. (8)).

$$\mu_x = \left(\mu_{maxX} X \left(\frac{Sg}{k_{Sg} + Sg} \right) \right) \left(1 - \left(\frac{But}{k_{But}} \right) \right) \left(1 - \left(\frac{Sg}{k_{Si}} \right) \right) \quad (8)$$

Additionally, to further reinforce the representation of biomass dynamics within the fermentation it was included a novel term for the representation of the cell's sporulation stage also based on the real-time pH in the medium and the implementation of an optimal growth pH value for the system (Eq. (9)) opposed to being a static constant which is far from being representative of the metabolic response of the culture.

$$\nu_{Spo} = - \left(0.12 - \left(\frac{e^{\left(\frac{pH - pH_c}{1.5} \right)}}{\left(1.5 \left(1 + e^{\left(\frac{pH - pH_c}{1.5} \right)} \right) \right)^2} \right) \right) X \quad (9)$$

Finally, it was assumed that glucose consumption was only driven by the uptake from the vegetative cells as a whole, and that spores weren't metabolically active (Eq. (10)).

$$\nu_{Sg} = \frac{\left(\mu_{maxX} X \left(\frac{Sg}{k_{Sg} + Sg} \right) \right) \left(1 - \left(\frac{But}{k_{But}} \right) \right) \left(1 - \left(\frac{Sg}{k_{Si}} \right) \right)}{Y_{XSg}} \quad (10)$$

2.2. Model development

The mathematical model for the IBE system was based on a classical mass balance approach under batch regime, and it was considered as a benchmark production plant by extending it to simulate continuous operation via the manipulation of the dilution rate as follows:

$$\frac{dSg}{dt} = D(Sg_a - Sg) - \nu_{Sg} \quad (11)$$

$$\frac{dX}{dt} = -DX + (\mu_x - \nu_{Spo}) \quad (12)$$

$$\frac{dBut}{dt} = -D(But) + \nu_{But} \quad (13)$$

$$\frac{dSb}{dt} = -D(Sb) + \nu_{Sb} - \nu_{USb} \quad (14)$$

$$\frac{dAct}{dt} = -D(Act) + \nu_{Act} - \nu_{UAct} \quad (15)$$

$$\frac{dIso}{dt} = -D(Iso) + \nu_{Iso} \quad (16)$$

$$\frac{dEt}{dt} = -D(Et) + \nu_{Et} \quad (17)$$

The kinetic model is composed of a set of seven differential equations (11)–(17) describing the corresponding mass balances for Glucose, Biomass, Butanol, Butyric Acid, Acetic Acid, Isopropanol and Ethanol respectively. D stands for the dilution rate of the reactor and $S_{g,i}$ is the glucose concentration within the feeding solution.

Now, for numerical simulation and further process control of the pH in the culture it's necessary to include an explicit state equation representing its dynamic behaviour in real time on the reaction vessel. To address such challenge the most accurate representation would need to make use of a proton mass balance approach for the modelling of this variable, yet the sheer difficulty to determine all the proton donors and acceptors into *Clostridium* growth media, which often are formulated with buffer solutions, and the need to apply a logarithmic function to transform such proton concentration data into pH makes the analytical procedure to calculate it and its experimental validation very computational intensive.

So, in an attempt to simplify, yet propose a valid equation for pH dynamics into the fermentation in this work it was proposed an equation that correlates the in-line concentration of acetic acid (the strongest in terms of pK_a) with the current pH of the medium via a proportional free parameter (Eq. (18)). This strategy also removes the need to apply logarithmic functions and its results can be directly incorporated into the previous kinetic rates to assert its effect over the metabolism of the culture.

$$\frac{dpH}{dt} = pH_{Act} \left(\frac{dAct}{dt} \right) \quad (18)$$

2.3. Parametric identification and numerical simulations

All the computational work was made using a PC equipped with an Intel® Xeon® E5450 processor at 3.2 GHz and 8 GB of DDR2 RAM at 1000 MHz (500 MHz DDR).

The parametric identification of the proposed model was made via the Marquardt algorithm into the software ModelMaker® 3.0.3. Experimental data was obtained from an IBE fermentation system reported by Lee et al. [10], which consisted of a stirred tank reactor of 3 L nominal volume working with 1.8 L of CGM medium with glucose as the main carbon source and inoculated with cells of *Clostridium acetobutylicum* pIPA3-Cm2, operated under batch regime.

For the statistical analysis of the goodness-of-fit there was applied a traditional determination coefficient over the simulated data versus the

experimental report and also a so-called E modelling efficient index [7]. E values can range from $-\infty$ to 1, where a value between $-\infty$ and 0 represent that the model is statistically worse as the mean value of a variable to represent the dataset, a value ranging from 0 to 0.5 indicates an “acceptable” performance and a value over 0.5 means that the model gets a “good” representation of the analysed variable (Eq. (19)).

$$E = 1 - \frac{\sum_1^n |Y - \bar{Y}|}{\sum_1^n |Y^* - \bar{Y}|} \in (-\infty, 1] \quad (19)$$

The numerical simulations of the system were made into the MATLAB® R2016a software, using the ODEsolver library's command ode15s to solve the system's set of differential equations over all the analysed operational conditions and regimes.

2.4. Parametric sensitivity analysis

Having identified the set of parameter values that maximized model fitting into the selected experimental values it was proceeded to conduct a local sensitivity analysis to determine the parameters that most influence the behaviour of the proposed structure [14].

Theoretically, differentiating the vector of state equations (x) respect to the parameter vector (θ), and assuming that the vector of the input variables (u) does not depend on θ , then:

$$\frac{d}{dt} \frac{dx}{d\theta} = \frac{\partial f(x, u, \theta)}{\partial x} \bigg|_{x=x(t, \theta^0)} \frac{dx}{d\theta} + \frac{\partial f(x, u, \theta)}{\partial \theta} \bigg|_{x=x(t, \theta^0)} \quad (20)$$

$$\frac{dS(t)}{dt} = \frac{\partial f(x, u, \theta)}{\partial x} \bigg|_{x=x(t, \theta^0)} S(t) + \frac{\partial f(x, u, \theta)}{\partial \theta} \bigg|_{x=x(t, \theta^0)} \quad (21)$$

where S are the sensitivity equations, defined as:

$$S(t) = S_j(t) = \frac{\partial x_i(t, \theta^0)}{\partial \theta_j}; \quad i = 1, \dots, n; \quad j = 1, \dots, p \quad (22)$$

And its maximum number is $n \times p$.

For analytical comparison between parameters, it's required to compute a relative sensitivity S^r , defined as:

$$S_j^r(t) = \theta_j^0 \left(\frac{\partial x_i(t, \theta^0)}{\partial \theta_j} \right) \neq 0; \quad i = 1, \dots, n; \quad j = 1, \dots, p \quad (23)$$

However, as the sensitivity equations are time-dependent, it's needed to calculate the integral term of the sensitivity equations to obtain a more comprehensive comparison of the numeric effect of the parameters over modelling performance as follows:

$$I = \int_0^t S(\tau) d\tau \quad (24)$$

where t is the fermentation time.

A relative sensitivity value for any parameter different from 0 indicates that the model would change its behaviour if the parameter changes its value even slightly but, if the relative sensitivity is equal to 0, then the model performance would remain unchanged no matter

Table 1
Parametric identification of the proposed model considering experimental data reported by Lee et al. [10].

Parameter	Value	Units	Parameter	Value	Units
k_{BAct}	9.4493 ± 0.6546	kg m ⁻³	ν_{maxEt}	0.01 ± 0.0037	h ⁻¹
k_{BAA}	2.0007 ± 0.9505	kg m ⁻³	ν_{maxIso}	0.6062 ± 0.1166	h ⁻¹
k_{Sb}	3.5 ± 1.2577	kg m ⁻³	ν_{maxSb}	0.995 ± 0.1068	h ⁻¹
k_{SS}	35 ± 4.0327	kg m ⁻³	μ_{maxx}	0.3796 ± 0.1615	h ⁻¹
k_{SSAct}	9.9973 ± 3.8765	kg m ⁻³	pH_{Act}	0.7 ± 0.1149	–
k_{SSEt}	15.9997 ± 1.9031	kg m ⁻³	pH_E	3.4997 ± 0.4535	–
k_{SSSb}	9.9924 ± 2.5388	kg m ⁻³	pH_{gp}	6.5 ± 0.2948	–
k_{Et}	327 ± 7.1754	kg m ⁻³	Y_{BAct}	0.7095 ± 0.1072	kg kg ⁻¹
ν_{maxAct}	0.4897 ± 0.0887	h ⁻¹	Y_{BAA}	0.9949 ± 0.2719	kg kg ⁻¹
ν_{maxEth}	0.995 ± 0.1549	h ⁻¹	Y_{SSg}	0.2339 ± 0.0473	kg kg ⁻¹

what the parameter value is.

3. Results

3.1. Model parametric identification

Table 1 summarizes the values obtained by the parametric identification of the selected model over experimental data reported by Lee et al. [10]. The validation of the model was made by simulation considering the following initial conditions: $X_0 = 0.75 \text{ g L}^{-1}$, $S_{G0} = 80 \text{ g L}^{-1}$, $But_0 = 0.01 \text{ g L}^{-1}$, $Sb_0 = 0.01 \text{ g L}^{-1}$, $Is_0 = 0.01 \text{ g L}^{-1}$, $Ac_0 = 0.01 \text{ g L}^{-1}$ and $Et_0 = 0.01 \text{ g L}^{-1}$, where the mathematical model represents the behaviour of the analysed variables with a global determination index $R^2 = 0.9931$.

The parametric uncertainty observed in some estimated values can be attributed to the fact that experimental data is measured with finite accuracy and only a subset of the state variables is accessible experimentally in an “in-line” manner, as samples must be obtained from the culture and processed off-line, this coupled with the inherent non-linearity nature of biological systems can be the main causes for such results [23]; however key parameters like the maximum specific growth rate (μ_{max}) are indeed within values reported into literature for *Clostridium* bacteria [15,17].

3.2. Model validation

Fig. 2 shows the obtained performance of the proposed model versus the experimental data reported by Lee et al. [10]. Also, includes the predicted trajectory of the in-line pH behaviour over time under open-loop operation, and said curve is comparable with experimental records of pH dynamics under the same operational conditions [1].

It's noteworthy to mention that even if the proposed model follows a classic Non-Structured approach for the construction of the equations, the proposed structure is capable of represent accurately the two characteristic growth stages of *Clostridium* life cycle, while also being evident that the metabolic shifting predicted by the model does coincide with the beginning of the sporulation of the culture and the ramping up of the solvent production rate at the 12–15 h mark.

Additionally, both the determination coefficients and modelling efficiency indexes for all the state variables show an adequate performance of the proposed structure against the experimental data, which helps to reinforce the validity of the value of the proposed parameters

Table 2

Determination coefficients and E indexes of the proposed model parametric identification versus experimental data reported by Lee et al. [10].

Variable	R^2	E
Glucose	0.9993	0.9809
Biomass	0.9599	0.8546
Butanol	0.9461	0.8475
Butyric Acid	0.9403	0.8097
Acetic Acid	0.7139	0.6897
Isopropanol	0.9364	0.8344
Ethanol	0.9489	0.9547
Global	0.9931	0.9805

(Table 2).

The low performance of the equation that represents the dynamic behaviour of acetic acid in fermentation can be attributed to the simulation results obtained from it during the time interval between 12 and 24 h of operation show values approximately 50% higher than those obtained experimentally. However, the curve generated by the proposed structure responds analogously to the phenomenon of acidogenesis, which is carried out in the fermentation during that same period of time. Additionally, it must be considered that the proposed model uses acetic acid dynamics as a basis to infer the behaviour of pH within the fermentation system, so this overestimation of the concentration made by the model makes it possible to compensate the lack of contribution of protons to the environment by acids not considered into the equation, such as butyric and lactic acids, where the latter is rarely quantified experimentally.

3.3. Parametric sensitivity

Figs. 3 and 4 show the results obtained for the determination of the local parametric sensitivity of the model over all the state equations and fermentation time, where it should be mentioned that the three proposed parameters for taking into account the effect of pH over the culture are indeed relevant for the adequate functionality of the structure. In this regard both pH_c and pH_{Act} values have a notorious influence into biomass growth, while the explicit pH-related parameter (pH_{op}) has not. Nonetheless, pH_{op} has a relevant paper into the adequate performance of the butyric acid equation, and as both butyric and acetic acids dynamics are key to the correct implementation of the acidogenic and solventogenic stage differentiation then its existence into the

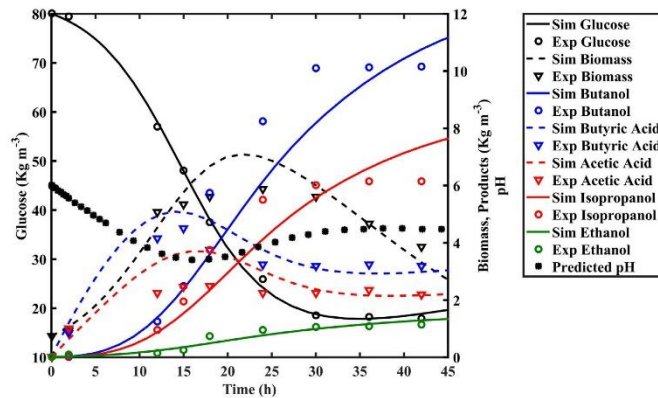


Fig. 2. Comparison between the simulated kinetics obtained by the proposed model versus experimental data of *Clostridium acetobutylicum* pIPA3-Cm2 under batch regime by Lee et al. [10].

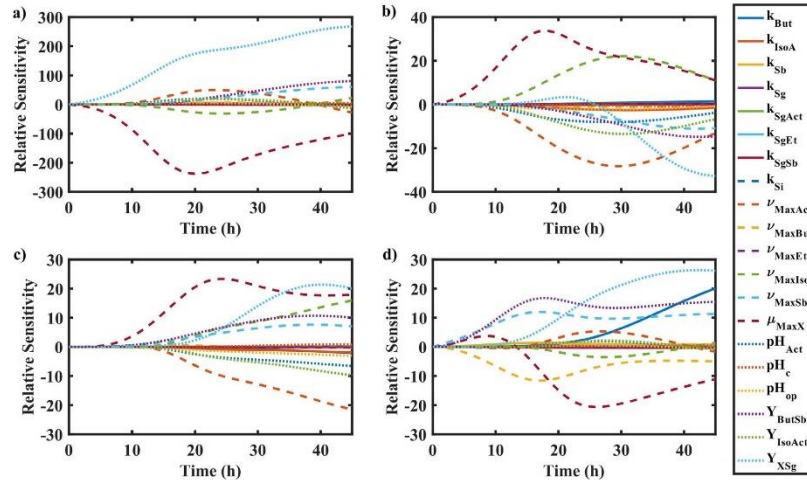


Fig. 3. Local parametric sensitivity of the proposed model obtained by the Fisher Information Matrix for (a) Glucose, (b) Biomass, (c) Butanol and (d) Butyric Acid dynamics under batch regime.

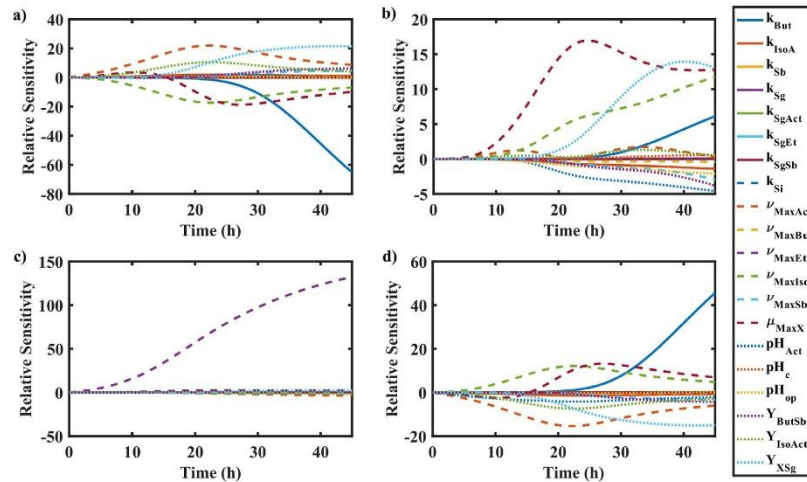


Fig. 4. Local parametric sensitivity of the proposed model obtained by the Fisher Information Matrix for (a) Acetic Acid, (b) Isopropanol, (c) Ethanol and (d) pH dynamics under batch regime.

proposed model is justified.

An interesting result to highlight from the calculation of the parametric sensitivity in Fig. 4 is the one observed for the dynamics of ethanol production, in which it is appreciated that the only parameter that sensitively affects its behaviour is its own specific production rate. This result is expected taking into account that within the initial considerations for the construction of the equations from Section 2.1 it is mentioned that this metabolite is constitutively generated throughout all the fermentation, so the sensitivity curves reported for this case maintain phenomenological congruence.

3.4. Numerical simulation and performance comparison

In order to determine the viability of the proposed structure to be used into predictive analysis of the IBE fermentation system there were made additional numerical simulations considering operational conditions outside the ones used to adjust the model.

It's important to remark that all the following results were made considering the single parameter set obtained in Section 3.1, there was no re-identification of the model parameters against any of the experimental data from all the analysed scenarios. This kind of

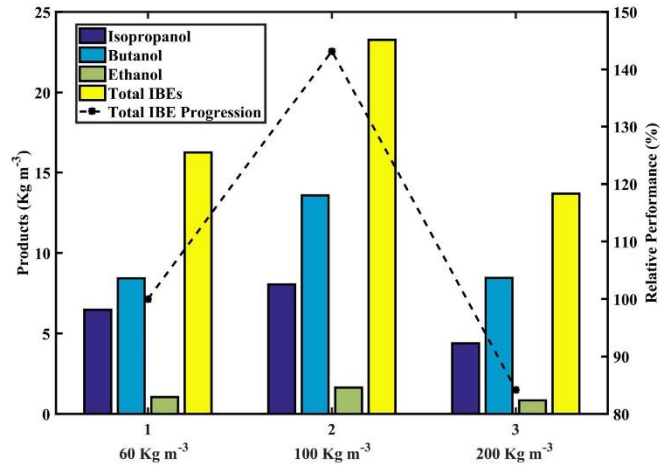


Fig. 5. Comparison between *Clostridium acetobutylicum* pIPA3-Cm2 fermentations under batch regime at different initial glucose concentrations and its relative performance against the data obtained with $S_{g0} = 60 \text{ kg m}^{-3}$.

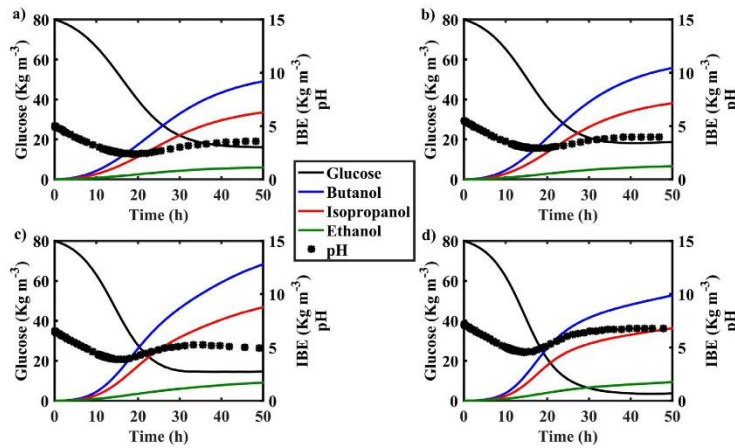


Fig. 6. Comparison between simulated *Clostridium acetobutylicum* pIPA3-Cm2 fermentations under batch regime at different initial pH values (a) $\text{pH}_0 = 5$, (b) $\text{pH}_0 = 5.5$, (c) $\text{pH}_0 = 6.5$ and (d) $\text{pH}_0 = 7$.

methodology is useful to determine the practical limits of application of the suggested structure and can help to the decision making process regarding its use for process control and optimization techniques.

The first analysis was made under batch regime but comparing the performance of the fermentation under different initial substrate concentrations. This essay in particular helps to clarify the importance of the k_{SI} parameter into the structure, as it was shown in Fig. 3 that it could be eliminated without affecting the overall performance of the model.

Fig. 5 shows the obtained IBE concentrations under either 60, 100 or 200 kg m^{-3} of initial glucose, where it can be appreciated that said operational condition does indeed affect the performance of the system and that the growth inhibition by substrate is being represented

adequately into the structure. An additional essay was made to compare the relative solvent production of the IBE system versus an ABE system reported by Qureshi et al. [16], where the overall behaviour pattern is followed by the simulation results, as the ABE system only reaches 72% of its total solvent concentration at 200 kg m^{-3} compared to the results at 60 kg m^{-3} , while the proposed model suggest that the performance hit at high glucose concentrations would be approximately -15% .

Now, to really determine if the proposed model can accurately account the pH effect over the IBE fermentation system there were made two additional simulation essays. First there was made a performance comparison under batch regime at different initial pH values but leaving the pH without control, whose results are shown into Fig. 6. Just as into the prior essay regarding initial substrate conditions initial pH

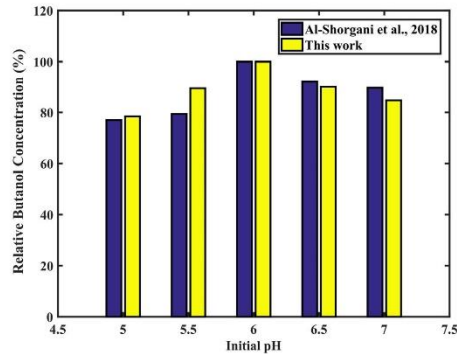


Fig. 7. Performance comparison between simulated *Clostridium acetobutylicum* pIPA3-Cm2 fermentations under different batch regime at different initial pH values versus experimental data reported by Al-Shorgani et al. [1].

manipulation produces similar behaviour. Also it was possible to determine the accuracy of the calculated value for the pH_{op} parameter, which coincides with the condition of maximum solvent production reported by Al-Shorgani et al. [1] as shown in Fig. 7.

The second numerical essay made to evaluate the pertinence of the proposed structure for the modelling of pH effect over IBE fermentation was an additional simulation under batch regime but now considering an initial pH of 6 and that said condition was maintained over the totality of the reactor operation. This numerical experiment was necessary to corroborate that the proposed structure was able to replicate the phenomenological behaviour of closed-loop *Clostridium* fermentation systems and to assert its capability to display the loss in solvent production capabilities due acid-crash. Fig. 8 shows that under such conditions the system arguments its biomass growth capabilities by approximately 250%, which is consistent with the results obtained by Wang et al. [24] for IBE fermentation systems operating at fixed pH values above 5.2 units. However, even with the increased biomass production the system loses about 45% of its solvent production capabilities, and such result is in the same magnitude order than the results obtained into literature for this kind of operation [30,1].

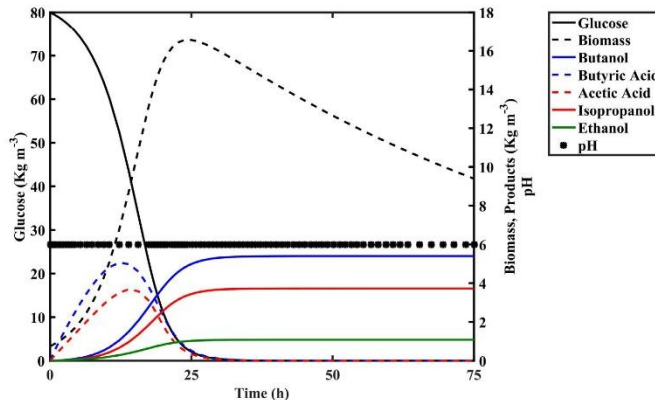


Fig. 8. Numerical simulation of the IBE fermentation system operating under batch regime considering closed-loop operation over the pH with set-point equal to 6 units.

Finally there was made a simulation experiment to verify that the proposed structure can be used not only under batch regime, but also for the determination of operating conditions under continuous regime, which is a required property to apply dynamic analysis techniques such as bifurcation analysis. For this simulation the initial conditions taken were the same reported by Lee et al. [10] but the operational parameters for continuous regime were taken from a study made by Yen and Li [29], which were $D = 0.05 \text{ h}^{-1}$ and $S_{G0} = 60 \text{ kg m}^{-3}$. Such conditions were selected as they're located at an adequate middle point where both washout and inhibition processes are non-existent experimentally.

The results of the continuous simulation are shown into Fig. 9, which indicated that the proposed model can be used to simulate continuous operation without incurring into mass conservation law violations due the added dilution term. Also it's interesting to note that the proposed pH differential equation does maintain adequate performance after the transition between batch and continuous regime, which in turn allows it to be further analysed under such regime.

4. Conclusions

The proposed mathematical model was able to reproduce the dynamic of the pH of the IBE fermentation system by *Clostridium acetobutylicum* pIPA3-Cm2 with the use of a relatively simple structure that works around the difficulty of establishing a proper proton balance within the culture medium due to its complexity. Additionally, the proposed sporulation module does indeed reflect the phenomenological behaviour of the culture by incorporating the newly available information for the in-line determination of pH dynamics within the reactor, which opens the possibility to enhance the fermentation performance via manipulation of said physicochemical variable.

Finally it should be noted that the versatility and accuracy of the numerical simulation results obtained based on this kinetic model under both batch and continuous regime and under the manipulation of several operational conditions such as the initial substrate concentration and both initial and dynamics of the pH within the reactor can be useful to use it as a guideline in the design, analysis, optimization and control of novel IBE producing systems coupled with either *in situ* or external recovery units, as it has enough robustness to withstand the perturbations imposed by the purification process and gives some flexibility to evaluate the performance of the system under variations of the operational conditions of such *downstream* process.

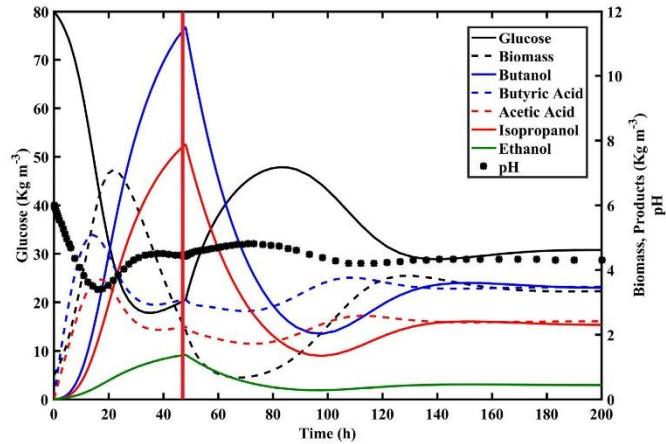


Fig. 9. Numerical simulation of the IBE fermentation system operating under continuous regime with the operational conditions reported by Yen and Li [29]: $D = 0.05 \text{ h}^{-1}$ and $S_{gl} = 60 \text{ kg m}^{-3}$. Vertical line indicates the transition from batch to continuous regime at $t = 45 \text{ h}$.

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Anexo 4: “Novel Feedback Control to Improve Biohydrogen Production by *Desulfovibrio alaskensis*”.

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Novel Feedback Control to Improve Biohydrogen Production by *Desulfovibrio alaskensis*

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Abstract: In this paper, a novel control algorithm to increase biohydrogen production in a continuous reactor using the sulphate-reducing bacteria *Desulfovibrio alaskensis* with lactate as carbon source is proposed. This work was conducted via numerical simulations, based on an experimentally corroborated kinetic model, considered as a benchmark of the system. A bifurcation analysis to identify the reactor's steady-state performance was done in order to identify feasible operating regions. The proposed controller cancels the upper bounds of the reactor, imposing a finite-time convergence to the selected set point. The closed-loop stability of the reactor is analysed via the dynamic of the regulation error. Finally, numerical experiments were conducted in order to compare the dynamic behaviour of the proposed closed-loop system versus its open-loop counterpart and a well-tuned classical PI controller one. The proposed methodology increases the hydrogen productivity controlling with a satisfactory performance the biomass concentration, which is considered as the control output.

Keywords: Biohydrogen, sulphate-reduction bacteria, *Desulfovibrio*, finite-time controllers, process intensification

1 Introduction

The incoming global energetic crisis, caused by the overexploitation of fossil fuels has motivated the search for viable replacements, both economically and environmentally speaking, for them. One of the most studied

alternatives in the last decade is hydrogen production, which could help to reduce greenhouse effect gaseous emissions coming from the transport sector, as its combustion only yields water vapour instead of carbon dioxide (Kotay and Das 2008).

Current developed industrial hydrogen production technologies are based on chemical and electrochemical processes such as hydrocarbon reforming and electrolysis, but they do possess the disadvantage of being highly energetic demanding and harsh with the environment (Holladay et al. 2009).

To overcome such problems research is being focused into the development of sustainable hydrogen production processes based on biological means, such as the so called biophotolysis, photofermentation and classical dark fermentation systems. However, both biophotolysis and photofermentation require a large light transfer area, produce a high amount and variety of sub-products. Also, hydrogenases which are the main enzymes that produce the higher amount of free H₂, are highly inhibited by the oxygen generated by photosynthesis (Hallenbeck 2009).

In contrast, dark fermentation processes using bacteria from the genus *Desulfovibrio* had attracted research interest because such systems have the additional advantage to be potentially used for the Chemical Oxygen Demand reduction in effluents from the dairy industry and also to treat wastewater containing high concentrations of heavy metals (Martins and Pereira 2013).

The main constraint to scale up the biohydrogen production system is that traditional batch operation reactors suffer by low productivity due the relatively slow growth cycle of the culture and the low hydrogen titer obtained at the end of the fermentation. Those shortcomings combined with the growth inhibitory effect of sulfide production within the reactor arise the need to generate operational strategies to reduce the impact of them into the process (Hallenbeck 2009). As an attempt to remedy this, several workarounds had been proposed, such as metabolic engineering (Jones 2008), co-cultures to also produce methane from organic acids derived from the main fermentation pathway (Keskin, Abo-Hashesh, and Hallenbeck 2011) or even optimization of process parameters like pH, temperature, cell load and carbon

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source concentration within the culture medium (Martins and Pereira 2013), but those strategies just had achieved productivities up to $0.084 \text{ mL H}_2 \text{ L}^{-1} \text{ h}^{-1}$ and yields just over 1.15% (v/w).

The implementation of continuous regime for biohydrogen production has shown promising results to improve the productivity of dark fermentation systems (Azwar, Hussain, and Abdul-Wahab 2014) and there are precedents in the literature about the use of anaerobic continuous stirred tank reactors (ACSTRs) for wastewater treatment using *Desulfovibrio vulgaris* (Humphries et al. 2004), therefore it's natural to try to evaluate the viability of the implementation of a system involving an ACSTR to improve the performance of the biological system.

Additionally, process control strategies can be seen as alternative proposals to alleviate the low conversion yields and instability of biological systems from a more macroscopic and immediate approach. The early successful application control strategy in process control is in the evolution of the PID controller and Ziegler-Nichols tuning method (Ziegler and Nichols 1942). Till nowadays, a high percent of the controllers implemented in the process industries are PID-type (Nikačević et al. 2012). However, as (i) the industrial demands (ii) the computational capabilities of controllers and (iii) complexity of systems under control increase, so the challenge is to implement advanced control algorithms (Spear 2005). Under this frame hyperbolic tangent laws have been proposed in order to overcome the PID controller's deficiencies (Xu and Yu 2010). Recently, alternative bounded functions have been considered for estimation and control purposes with satisfactory performance, particularly for their implementation to biological processes (Aguilar-López et al. 2010; López-Pérez, Neria-González, and Aguilar-López 2013), without having to resort to the use of more complex algorithms or strategies like neural networks, fuzzy logic or model predictive control (Santín, Pedret, and Vilanova 2015), which do require either an accurate and robust plant model or a high level of instrumentation and computational power to achieve their goal.

Therefore this work proposes to evaluate the behaviour of a theoretical ACSTR fermentation system for biohydrogen production by *Desulfovibrio alaskensis* 6SR via simulation analysis based on a novel homogeneous Non Structured, experimentally corroborated, kinetic model. Bifurcation analysis was done in order to select feasible operating regions and then it is proposed a novel non-linear control algorithm in order to keep an adequate biomass concentration within the reactor to indirectly allow the bioreactor to increase its biohydrogen productivity. The results of this analysis can serve as a base to

evaluate the technical and economic viability of the scale up of this kind of biofuel producing systems.

2 Methodology

2.1 Kinetic modelling

Unstructured kinetic models are frequently employed for the characterization of bacterial growth, substrate consumption and product formation in bioprocesses. Currently both Gompertz and Luiking-Piret type structures have been used to describe the dynamics of H_2 production of biological systems (Koku et al. 2003; Das and Veziroğlu 2001), however these structures consider the process just as a dependence of either time of biomass growth respectively, while in reality biohydrogen production by *Desulfovibrio* bacteria is generally decoupled from the primary metabolism of the culture (Walker et al. 2009; Martins and Pereira 2013) making them inaccurate and thus, unsuitable for process control strategies. Novel developed mathematical models that consider more biochemical and genetic information are being developed to overcome such troubles, but generally these are complex enough to make them unsolvable analytically.

Therefore, a homogeneous Non-Structured mathematical model was developed to describe the kinetics of H_2 production in batch systems, then the model was considered as a benchmark production plant by extending it to simulate continuous operation. The prior assumption is valid as long as the reactor can be operated near perfect mixing conditions, which can be scaled up reliability at least up to pilot level as validated experimentally for similarly modelled systems (López and Borzacconi 2010; Sforza, Enzo, and Bertucco 2014; Ariyajaroenwong et al. 2016):

$$\frac{dX}{dt} = (\mu - Kd)X - (D^*X) \quad (1)$$

$$\frac{dLac}{dt} = (D(Lac_{in} - Lac)) - \left(\frac{\mu X}{Y_1}\right) - (r_{H_2} X^* Y_5) \quad (2)$$

$$\frac{dSO}{dt} = (D(SO_m - SO)) - \left(\frac{\mu X}{Y_2}\right) \quad (3)$$

$$\frac{dS^-}{dt} = \left(\frac{\mu X}{Y_3}\right) - (D^*S^-) \quad (4)$$

$$\frac{dAc}{dt} = \left(\frac{\mu X}{Y_4}\right) - (D^*Ac) \quad (5)$$

$$\frac{dH}{dt} = (r_{H_2}X) - (D^*H) \quad (6)$$

Where:

$$\mu = \mu_{\max} \left(\frac{SO}{SO + K_S} \right) \left(\frac{Lac}{Lac + k_{Lac}} \right) \left(1 - \frac{S^-}{K_{is}} \right)^n \quad (7)$$

$$r_{H_2} = r_{\max} \left(\frac{Lac}{Lac + k_{LH}} \right) \left(1 - \frac{S^-}{K_{is}} \right)^n \quad (8)$$

The proposed kinetic model is composed of 6 ordinary differential eqs (1)–(6) that describe the dynamics of biomass (X), lactate (Lac), sulphate (SO), sulphide (S^-), acetate (Ac) and hydrogen (H) concentrations in the reactor respectively. To describe the kinetics of cell growth, a Levenspiel-type expression was considered into the model (7), since it is known that bacteria of the genus *Desulfovibrio* exhibit growth inhibition due accumulation of sulphide in the culture medium (Okabe et al. 1995), while the loss of cell viability it's represented by the addition of an specific death constant (Kd). It must be noted that eq. (8) represents an independent kinetic rate for hydrogen production and also accounts the inhibitory effect of sulphide accumulation, as it does lower the redox potential of the culture medium (Martins and Pereira 2013).

The parametric identification of the proposed model was made via the Marquardt algorithm into the software ModelMaker[®] 3.0.3. Experimental data was obtained from a batch system using *Desulfovibrio alaskensis* 6SR performed in anaerobic conditions at 37 °C for 168 h (López-Pérez, Neria-González, and Aguilar-López 2013). It consisted in 80 serum bottles (250 mL) with 45 mL of modified Postgate medium with lactate as carbon source and inoculated with 10% (v/v) of a 36 h old seed culture. A set of 10 bottles was taken every 24 h for analyses of all the modelled variables.

2.2 Numerical simulations

All the numerical simulations were made into a PC equipped with an Intel[®] Core[®] i5 560 M processor and 4 GB of RAM into the MATLAB[®] 2013b software, first using the ODE solver library's command ode15s to solve the system's set of differential equations at an undefined time-span assuming continuous mode operation until steady state was observed, then for bifurcation analysis the system was solved employing the Matcont 5p0 toolbox for the generation of the phase diagrams considering the reactor's dilution rate (D) and sulphate feeding concentration (SO_{in}) as bifurcation parameters.

2.3 Proposed controller

The proposed controller eq. (8) considers the biomass concentration as measured output, such that it can be on-line determined from the reactor via an optical density determination, and the dilution rate (D) as the control input. The proposed controller's structure is giving by eq. (8) as:

$$D = u = k_1 \left(e^{-\frac{X}{k_2}} - k_2 \right) \text{ where } e^{-\frac{X}{k_2}} = X - X_{sp} \quad (9)$$

The main advantage of the proposed structure is that it can cancel the upper bounds of the bioreactor and impose a finite-time convergence to the set point as is demonstrated analytically in the Theory subsection. The tuning criteria is based on the basic knowledge of the reaction system, as the product $k_1 k_2$ can be approximated for biological systems to $\max(\mu)$ divided by $\max(D)$, so the exact functions describing the plant's behaviour doesn't need to be explicitly known *a priori*.

3 Theory

3.1 Sketch of proof of the closed-loop system's stability

Considering the system as the following:

$$\dot{x} = f(x) + g(x)u \quad (10)$$

Where:

$$f(x) = \begin{bmatrix} (\mu - Kd)X \\ -\left(\frac{\mu X}{Y_1}\right) - (r_{H_2} X^* Y_5) \\ -\left(\frac{\mu X}{Y_2}\right) \\ \left(\frac{\mu X}{Y_3}\right) \\ \left(\frac{\mu X}{Y_4}\right) \\ (r_{H_2} X) \end{bmatrix}$$

And:

$$g(x) = \begin{bmatrix} -X \\ Lac_{in} - Lac \\ SO_{in} - SO \\ -S^- \\ -Ac \\ -H \end{bmatrix}$$

Considering that the vector field that define the bioreactor dynamic is bounded, then it is defined an upper bound F and G such that:

$$f(x) \leq F; g(x) \leq G \tag{11}$$

Then:

$$\dot{x} = \dot{e} = f(x) + g(x)k_1 \left(e^{\frac{1}{m}} - k_2 \right) \tag{12}$$

Under the assumption that the set point for biomass (X_{sp}) is constant (regulation case) and applying the Cauchy-Schwartz inequality to eq. (11):

$$\dot{e} \leq F + G \left[k_1 \left(e^{\frac{1}{m}} - k_2 \right) \right] \tag{13}$$

If it is chosen that:

$$k_1 k_2 \cong G^{-1} F \tag{14}$$

Then:

$$\dot{e} \leq k_1 G e^{\frac{1}{m}} \tag{15}$$

The above inequality induce a finite time convergence of the regulation error, where parameter $m > 1$ should be an odd integer. Then the solution of inequality eq. (14) is,

$$e \leq \text{sign}(e_0) \left(e_0 - Gk_1 \frac{t}{m} \right)^m \tag{16}$$

At steady state ($e(t) = 0$),

$$t \geq m \frac{e_0^{\frac{1}{m}}}{Gk_1} \tag{17}$$

Then finite-time convergence is given by,

$$t_{ft} = m \frac{e_0^{\frac{1}{m}}}{Gk_1} \tag{18}$$

4 Results and discussion

Table 1 summarizes the values obtained by the results of the parametric identification of the proposed model versus experimental data obtained by López-Pérez, Neria-González, and Aguilar-López (2013), which consider the following initial conditions: $X_0 = 20 \text{ mg L}^{-1}$, $Lac_0 = 464 \text{ mg L}^{-1}$, $SO_0 = 5,655 \text{ mg L}^{-1}$, $S^-_0 = 29 \text{ mg L}^{-1}$, $Act_0 = 0.01 \text{ mg L}^{-1}$ and $H_0 = 0 \text{ mL L}^{-1}$, and a fermentation time of 160 h (for the physical meaning of each parameter please refer to the Nomenclature section). Figure 1 shows the validation results via simulation, were the model represents the dynamics of the modelled variables with a linear correlation coefficient $r^2 = 0.9948$ and a p -value < 0.001 , which ensures there is not a significant difference between the predicted behaviour described by the proposed model and the experimental data (Sellke, Bayarri, and Berger 2001). The high parametric uncertainty observed in some estimated values can be attributed to the fact that experimental data is measured with finite accuracy and only a subset of the state variables is accessible experimentally in an “in-line” manner, as samples must be obtained from the culture and processed offline, this coupled with the inherent non-linearity nature of biological systems can be the main causes for such results (Vanlier et al. 2013); however key parameters like the maximum specific growth rate (μ_{max}) are indeed within values reported into literature for *Desulfovibrio* bacteria (Badziong and Thauer 1978; Sani, Peyton, and Brown 2001; López-Pérez, Neria-González, and Aguilar-López 2013). The experimental biohydrogen productivity for the batch system was $0.1202 \text{ mL L}^{-1} \text{ h}^{-1}$.

To determine the steady state of the system under continuous regime in open loop, simulations considering operational conditions for D , SO_m and Lac_m of 0.01 h^{-1} , $1,250 \text{ mg L}^{-1}$ and 500 mg L^{-1} , respectively and the same initial values for the six evaluated variables as the ones

Table 1: Parametric identification of the proposed model considering experimental data.

Parameter	Value	Units	Parameter	Value	Units
Kd	$0.0082 \pm 4.41e^{-5}$	h^{-1}	r_{max}	0.0101 ± 0.017	$\text{mg L}^{-1} \text{ h}^{-1}$
$Klac$	0.1 ± 4.3067	mg L^{-1}	Y_1	1.5 ± 0.013	mg mg^{-1}
klh	345.39 ± 139.62	mg L^{-1}	Y_2	0.1434 ± 0.005	mg mg^{-1}
Kp	$1,253.36 \pm 123.86$	mg L^{-1}	Y_3	1.0214 ± 0.003	mg mg^{-1}
ks	$4,681.08 \pm 241.86$	mg L^{-1}	Y_4	1.777 ± 0.006	mg mg^{-1}
μ_{max}	0.3 ± 0.676	h^{-1}	Y_5	0.0390 ± 0.066	mg mL^{-1}
n	4.95 ± 1.7	Dimensionless			

Source: Reported by López-Pérez, Neria-González, and Aguilar-López (2013).

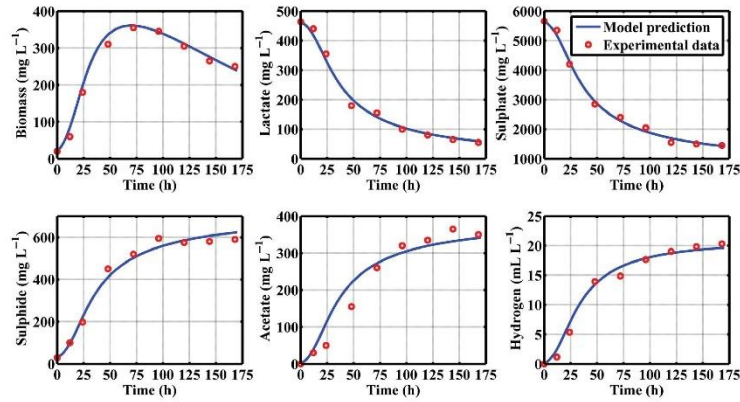


Figure 1: Dynamics predicted by the proposed kinetic model under batch mode operation versus experimental data. Source: Reported by López-Pérez, Neria-González, and Aguilar-López (2013).

reported for batch regime operation were made. The results indicated that the system reaches steady state condition after 1,000 h of fermentation and the hydrogen concentration and productivity were 21.62 mL L⁻¹ and 0.2162 mL L⁻¹ h⁻¹, which then was used as a base for the comparison of the performance of the system under closed-loop operation.

With the results obtained with the prior simulations there was applied a bifurcation analysis over the dilution rate to identify operational range values for the controller to avoid washout into the reactor. The results of said procedure are showcased into Figure 2, where it was observed that the reactor's washout rate (D_w) was equal to 0.087 h⁻¹.

It must be noted that even if the correlation between D and H_2 is always inversely proportional, the behaviour

of the biomass within the reactor against the dilution rate is not. There are two regions which can be identified in the diagram showcased in Figure 2 that suggest there is a local maximum for biomass concentration at $D = 0.015$ h⁻¹, which corresponds to an $X = 118.5$ mg L⁻¹ and as the biomass is being considered as the controlled variable said behaviour can affect the performance of the closed-loop system as there are two different dilution rates that can bring the biomass concentration to the desired set-point value. For that issue, and taking into account that the scope of this work is to enhance hydrogen productivity then the controller was bounded to operate between the interval $D = (0.015, D_w)$, as higher dilution rates allow for a higher productivity of the system and allows to justify the use of an ACSTR versus the traditional batch culture.

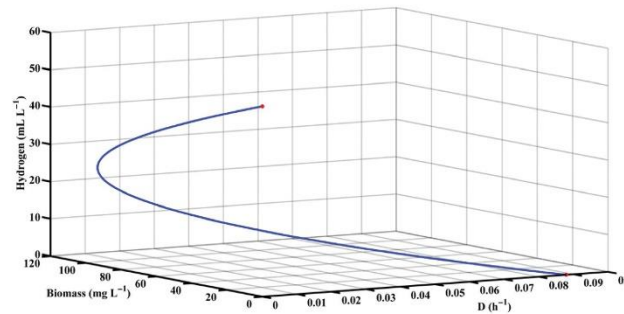


Figure 2: Bifurcation diagram portraying the dynamics of biomass and hydrogen concentration at steady state considering the reactor's dilution rate as bifurcation parameter.

As an additional study, there was made use of the bifurcation analysis to find the theoretically maximum sulphate concentration that the biological system could handle without compromising its performance due sulphide accumulation and to efficiently process the incoming carbon source into hydrogen production. The results obtained by said study indicated that the best performance was achieved when SO_m was equal to $2,215 \text{ mg L}^{-1}$.

Table 2 indicates the obtained values for the proposed controller tuning, using the considerations mentioned into the Theory subsection eq. (13), while for the selection of the set-point for biomass concentration further simulations were made considering values ranging from 69 to 118.5 mg L^{-1} , as those correspond to the steady state predicted by the model for the dilution rate interval from 0.015 to 0.087 h^{-1} , respectively, in order to assess which of these could be maintained into the reactor to overweight the inhibitory effect of sulphide accumulation into the vessel, and thus indirectly improve the final hydrogen titer of the fermentation. Additionally, the control effort was monitored by plotting the punctual dilution rate over time to determine the controller's stability and convergence time to the proposed set-point.

Table 2: Summary of the values obtained for the tuning of the proposed controller using the considerations made into the Theory subsection and heuristic criteria.

Parameter	Value	Units
k_1	0.055	$\text{mg L}^{-1} \text{ h}^{-1}$
k_2	0.01	mg L^{-1}
m	3	----
X_{sp}	100	mg L^{-1}

In order to validate the performance of the proposed feedback structure there were made comparisons versus a PI controller tuned via the Internal Model Control heuristic criteria (Rivera, Morari, and Skogestad 1986), whose results appear in Table 3

Table 3: Summary of the values obtained for the tuning of a PI controller using the IMC criteria (Rivera, Morari, and Skogestad 1986).

Parameter	Value	Units
k_c	0.33	h^{-1}
t_i	571.875	h
ΔU	0.01	h^{-1}

Figure 3 demonstrates that the proposed controller stabilises the biomass concentration into the proposed set-point in less than 285 h, versus the PI controller that requires at least 4,000 h to attain such condition, as even if the proposed structure causes a higher overshoot of the controlled variable it also does converge faster. Furthermore an integral time absolute error (ITAE) analysis (Smith and Corripio 1985) was performed to compare the proposed structure behaviour with the application of the PI structure and tracking of the ITAE versus time is shown in Figure 4. Once again, it's seen that the proposed structure attains lower ITAE values than the PI one by almost one magnitude order and such behaviour is maintained throughout the temporal spectrum evaluated in this essay (5,000 h).

To evaluate the system's stability, monitoring of the control effort was made, which consisted in the tracking of the dynamics of both the non-controlled variables (Figure 5) and of the dilution rate over time (Figure 6). In both cases it's shown that the control effort does indeed stabilize without visible delay after the biomass

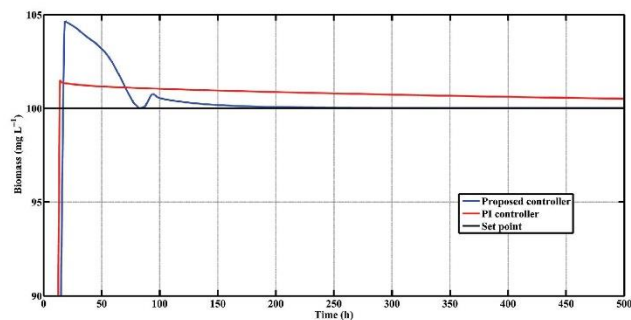


Figure 3: Comparison of the biomass (controlled variable) dynamic under closed-loop operation between the proposed controller and a PI one.

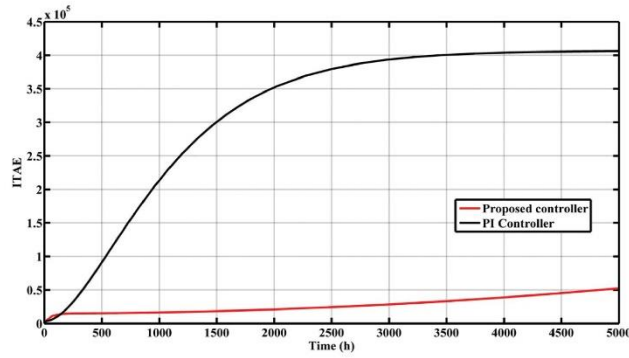


Figure 4: Evolution of the Integral Time Absolute Error performance index over time of both the proposed feedback structure and a PI controller.

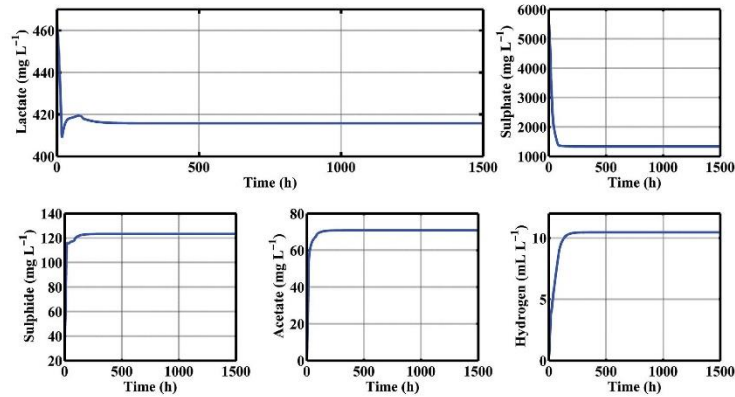


Figure 5: Dynamic of the non-controlled variables (Sulphate, Sulphide, Acetate, Lactate and Hydrogen) of the system under closed-loop operational regime using the proposed controller.

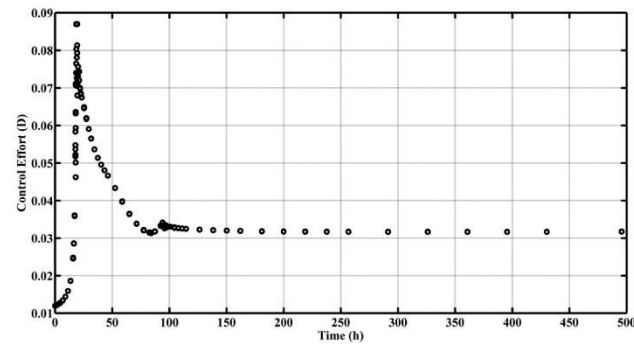


Figure 6: Dynamic of the control effort (dilution rate) of the biohydrogen production system under closed-loop operational regime using the proposed controller.

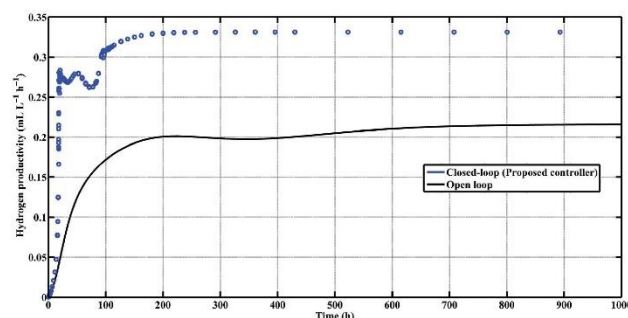


Figure 7: Comparison of the dynamic of biohydrogen productivity between the proposed ACSTR under open-loop operation (continuous line) and under closed-loop (discontinuous line).

Table 4: Comparison of the biohydrogen productivities by *Desulfovibrio* bacteria reported into literature.

Microorganism	Substrate	Type of reactor	H ₂ productivity (mL L ⁻¹ h ⁻¹)	Reference
<i>Desulfovibrio caledoniensis</i>	Lactate	Microbial Electrolysis Cell (MEC)	0.1205	Yu et al. (2011)
<i>Desulfovibrio vulgaris</i>	Lactate	Batch Stirred Tank Reactor	0.084	Martins and Pereira (2013)
<i>Desulfovibrio alaskensis</i> 6SR	Lactate	Batch Stirred Tank Reactor	0.1202	López-Pérez, Neria-González, and Aguilar-López (2013)
<i>Desulfovibrio alaskensis</i> 6SR	Lactate	Anaerobic Continuous Stirred Tank Reactor (Open-loop)	0.2162	This work
<i>Desulfovibrio alaskensis</i> 6SR	Lactate	Anaerobic Continuous Stirred Tank Reactor (Closed-loop)	0.3312	This work

set-point was reached and that the remaining variables do exhibit a stable behaviour, which could be inferred due the absence of critical points predicted by the model into the bifurcation analysis stage.

Finally, further simulations made considering the closed-loop system demonstrated this operational regime provided the best hydrogen productivity compared with the ones obtained by the open-loop simulation and the closed-loop one under PI control (Figure 7), even if the concentration a steady state is lower. As hydrogen can be easily recovered from the reactor then said parameter isn't critical for the design of the downstream processes as opposed to other biofuels like bioethanol or butanol (Das and Veziroğlu 2001). Also, Table 4 presents a summary of experimentally obtained biohydrogen productivities up to date versus the theoretically ones obtained in this work, which further reinforces the viability of the implementation of the proposed production scheme.

5 Conclusions

The proposed control algorithm applied to a biohydrogen ACSTR fermentation system using the sulphate-reducing bacteria *Desulfovibrio alaskensis* 6SR can improve its productivity from 0.1202, obtained experimentally under batch regime, up to 0.3312 mL L⁻¹ h⁻¹, which represents an increase of 56.16% over the open loop system and also 394% over the maximum reported for a traditional batch process using lactate as carbon source (Martins and Pereira 2013). In turn, it was found that the proposed feedback structure can be easily tuned and it does provide better performance than a traditional PI controller, which is backed up by the dynamics of the ITAE performance index. Said results shows the potential of the implementation of a closed-loop ACSTR system for the scaling up of biohydrogen production processes outside laboratory.

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Nomenclature

Symbol	Description	Units
μ_{max}	Maximum bacterial specific growth rate	h^{-1}
Ac	Acetate concentration into the reactor	$mg L^{-1}$
e	Absolute estimation error	----
H	Hydrogen concentration into the reactor	$mL L^{-1}$
$k1$	Proportional gain of the proposed controller	----
$k2$	Proposed controller tuning variable	----
Kd	Cell death constant	h^{-1}
Kis	Levenspiel's product inhibition constant	$mg L^{-1}$
$klac$	Lactate affinity constant for biomass growth	$mg L^{-1}$
kIh	Lactate affinity constant for hydrogen production	$mg L^{-1}$
Ks	Substrate affinity constant	$mg L^{-1}$
Lac	Lactate concentration into the reactor	$mg L^{-1}$
Lac_{in}	Lactate concentration of the feeding solution	$mg L^{-1}$
m	Proposed controller tuning variable	----
n	Levenspiel's product inhibition exponent	----
r_{max}	Maximum specific hydrogen production rate	$mg L^{-1} h^{-1}$
S	Sulfide concentration into the reactor	$mg L^{-1}$
SO	Sulphate concentration into the reactor	$mg L^{-1}$
SO_{in}	Sulphate concentration of the feeding solution	$mg L^{-1}$
X	Biomass concentration	$mg L^{-1}$
Y_1	Biomass yield over lactate	$mg mg^{-1}$
Y_2	Biomass yield over sulphate	$mg mg^{-1}$
Y_3	Sulfide yield over biomass	$mg mg^{-1}$
Y_4	Acetate yield over biomass	$mg mg^{-1}$
Y_5	Hydrogen yield over lactate	$mg mL^{-1}$

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