CENTRO INTERNACIONAL DE ESTUDOS
DE DOUTORAMENTO E AVANZADOS
DA USC (CIEDUS)

TESE DE DOUTORAMENTO

SOLVENT-BASED APPROACHES TO EVALUATE THE ABE EXTRACTIVE FERMENTATION

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Solvent-based approaches to evaluate the ABE extractive fermentation

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Solvent-based approaches to evaluate the ABE extractive fermentation

D. Juan Lema Rodicio, Catedrático de Enxeñería Química, e D. Thelmo Lu-Chau, investigador contratado doutor

INFORMA/N:

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INDEX

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Abbreviations & Acronyms

1G: first generation

2G: second generation

2B1O: 2-butyl-1-octanol

3G: third generation

ABE: Acetone-Butanol-Ethanol

ACC: American Type Culture Collection

AUC: area under the curve

C: carbon

CAPEX: CAPital EXpenditures

CDW: cell dry weight

CECT: Colección Española de Cultivos Tipo

DFCF: Discounted free cash flow

DS: Downstream section

EH: Enzymatic hydrolysis

EPS: Extracellular polymeric substance

FC: flow cytometry

FL1: fluorescence signal

FSC: forward scattering

HC: hydrolysate of cellulose

HC-Lac: hydrolysate of cellulose, prior laccase detoxification treatment

HCB: black hydrolysate of cellulose (without centrifugation)

HCB-Lac: black hydrolysate of cellulose, prior laccase detoxification treatment

LLE: Liquid-Liquid Extraction

GC: gas chromatography

ISPR: In Situ Product Recovery

K: Partition coefficient

LCB: Lignocellulosic biomass

MBSP: Minimum butanol selling price

NPV: Net present value

OA: Oleyl Alcohol

OD: optical density

OPEX: Operational Expenditures

PI: Propidium Iodide

PO: Pomace Oil

RM: Raw Materials

SEL: Selectivity

SFO: Sunflower Oil

SSC: side scattering

TEM: Transmission electron microscopy

VO: Vegetable Oil

WACC: Weight average capital cost

WWT: Waste Water Treatment

Resumo

O principal obxectivo desta tese é a mellora da fermentación Acetona-Butanol-Etanol (ABE) extractiva, aplicando enfoques baseados no disolvente. O cambio climático e a procura dunha independencia dos recursos fósiles aceleran, a día de hoxe, o desenvolvemento de procesos sostibles, a partir de materiais renovábeis. Dadas as súas múltiples aplicacións, o biobutanol ten un papel relevante neste contexto: ben coma biocarburante avanzado ou ben coma base química.

Durante a fermentación ABE, o biobutanol é producido por bacterias anaerobias do xénero Clostridia. Este proceso operou a escala industrial antes de ser progresivamente remprazado pola petroquímica en plena expansión, a finais do século XX. A reindustrialización da fermentación ABE vese freada por uns altos custos de produción, ligados a unha forte inhibición por produto e a un rendemento intrínseco limitado. A redución destes custos depende en gran medida da aplicación de técnicas que permitan integrar a eliminación do produto tóxico. Nesta tese avaliouse a aplicación *in situ* dunha extracción líquido-líquido sobre o proceso ABE global así como a resposta do microorganismo ós disolventes.

Primeiramente, levouse a cabo una selección global multicriterio de disolventes de extracción, en base ás súas propiedades físicas para a separación do butanol (coeficiente de reparto, selectividade) e á resposta biolóxica por parte do microorganismo (biocompatibilidade, biodisponibilidade).

A continuación, analizáronse as cinéticas da fermentación ABE extractiva con disolventes de características opostas. Ademais, aplicouse a citometría de fluxo para monitorizar o ciclo celular do microorganismo e cuantificar a dinámica da poboación microbiana. Cunha metodoloxía novidosa baseada na evolución do cultivo heteroxéneo, formulouse un modelo matemático de fermentación.

Tomando como punto de partida os resultados experimentais, realizouse unha avaliación técnico-económica comparativa do proceso global integrado, en base ó disolvente empregado e ó modo de operación (*batch/fed-batch*). Por último, validouse o uso dun hidrolizado procedente do proceso *Organosolv* como fonte de carbono, integrando desta forma o proceso no concepto de biorrefinería.

Os resultados desta tese proporcionan un avance na comprensión dos fenómenos de interacción disolvente-microorganismo durante a fermentación ABE extractiva. Igualmente, obtense información relevante sobre a repercusión dos devanditos efectos no proceso integrado global.

Palabras clave: ABE, *Clostridium acetobutylicum*, extracción líquido-líquido, fermentación extractiva

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Resumen

El principal objetivo de esta tesis es la mejora de la fermentación Acetona-Butanol-Etanol (ABE) extractiva, aplicando enfoques basados en el disolvente. El cambio climático y la búsqueda de una independencia de los recursos fósiles aceleran hoy en día el desarrollo de procesos a partir de material renovable. Dadas sus múltiples aplicaciones, el biobutanol juega un papel clave en este contexto: bien como biocarburante avanzado o bien como base química para la industria.

Durante la fermentación ABE el biobutanol es producido por bacterias anaerobias del género Clostridia. Este proceso operó a escala industrial antes de ser progresivamente reemplazado por la petroquímica a finales del siglo XX. Su reindustrialización se ve frenada por unos altos costes de producción, ligados a la inhibición por producto y al bajo rendimiento intrínseco. La reducción de estos costes depende en gran medida de la aplicación de técnicas que permitan integrar la eliminación del producto tóxico. En esta tesis se evaluó la aplicación de una extracción líquido-líquido *in situ* sobre el proceso global, así como la respuesta del microorganismo a los disolventes.

En primer lugar se llevó a cabo una selección global de disolventes de extracción, en base a sus propiedades físicas para la separación del butanol (coeficiente de reparto, selectividad) y a la respuesta biológica por parte del microorganismo (biocompatibilidad, biodisponibilidad).

A continuación se analizaron las cinéticas de la fermentación ABE extractiva con disolventes de naturaleza opuesta. Además, se aplicó la citometría de flujo para monitorizar el ciclo celular del microorganismo y cuantificar la dinámica de la población microbiana. Con una metodología novedosa, basada en la evolución del cultivo heterogéneo, se planteó y validó un modelo matemático de fermentación.

Tomando como punto de partida los resultados experimentales, se realizó una evaluación técnico-económica comparativa del proceso global integrado, en base al disolvente utilizado y al modo de operación (*batch/fed-batch*). Por último, se validó el uso de un hidrolizado procedente del proceso *organosolv* como fuente de carbono, integrando de esta forma el proceso en el concepto de biorrefinería.

Los resultados de esta tesis proporcionan un avance en la comprensión de los fenómenos de interacción disolvente – microorganismo durante la fermentación ABE extractiva. Igualmente, se obtiene información relevante sobre la repercusión de dichos efectos en el proceso integrado global.

Palabras clave: ABE, *Clostridium acetobutylicum*, extracción líquido-líquido, fermentación extractiva

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Abstract

The main objective of this thesis is to enhance the Acetone-Butanol-Ethanol (ABE) extractive fermentation by the application of solvent-based approaches. Climate change and the search for an independence from fossil resources accelerates the development of sustainable processes, based on renewable material. Given its multiple applications, biobutanol plays a key role in this context: as an advanced biofuel, or as a platform molecule in the chemical industry.

During ABE fermentation, biobutanol is produced biologically by anaerobic bacteria of the Clostridia genus. This process was operated on an industrial scale before being progressively replaced by petrochemistry at the end of the 20th century. The reindustrialization of ABE fermentation is hampered by significant production costs, linked to high inhibition by product and limited intrinsic yield. The reduction of these costs depends to a large extent on the effective application of integrated toxic product removal techniques. In this thesis, the in-situ application of a liquid-liquid extraction on the global ABE process and the response of the producing microorganism to the presence of solvents were evaluated.

First, a multi-criteria global selection of extraction solvents for ABE fermentation was carried out, based on its physical properties for the separation of butanol (partition coefficient, selectivity) and the biological response to the microorganism (biocompatibility, bioavailability).

Next, the kinetics of extractive ABE fermentation were analyzed with solvents of opposite nature. Flow cytometry was applied to monitor the cell cycle of the microorganism and quantify the microbial population dynamics. A mathematical model based on the evolution of the heterogeneous culture inside the bioreactor, was proposed and validated.

Considering the experimental results, a comparative techno-economic evaluation of the integrated global process was carried out, based on the solvent used and the applied mode of operation (*batch / fed-batch).* Finally, the use of a hydrolyzate from the *organosolv* process as a carbon source was validated, thus integrating the process in the concept of biorefinery.

The results of this thesis provide an advance in the understanding of the solvent-microorganism interaction phenomena in a biphasic reactor for extractive ABE fermentation. Likewise, relevant information is obtained on the impact of these effects on the global integrated process.

Key words: ABE, *Clostridium acetobutylicum*, liquid-liquid extraction, extractive fermentation

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Resumen extendido

A día de hoy la sociedad se enfrenta a una crisis global sin precedentes que tiene como factores principales un crecimiento poblacional incesante, una escasez cada vez mayor de recursos y una urgencia medioambiental inminente, con una de sus más graves expresiones en el cambio climático. Como consecuencia, existe una motivación para redefinir el modelo productivo, y son varias las estrategias, a diferentes niveles, para asentar los cimientos de la llamada bioeconomía. Se busca así reemplazar la síntesis tradicional de carburantes, materiales y bases químicas, a partir de petróleo y gas, por procesos alternativos que dependan de fuentes renovables sostenibles. Además de dar cabida a nuevos procesos, productos y aplicaciones en esta transición, parece igualmente sensato hacer resurgir bioprocesos antiguamente implantados, que han sido progresivamente reemplazados por la industria petroquímica.

Un buen ejemplo es el caso de la fermentación ABE (Acetona-Butanol-Etanol), que se posicionó como la segunda fermentación industrial de mayor importancia durante gran parte del siglo XX, y que ha sido objeto de un renovado interés científico e industrial durante las últimas dos décadas. El biobutanol, producto mayoritario de esta fermentación, podría ser una pieza clave en la definición de futuras biorrefinerías, dadas sus múltiples aplicaciones. Por un lado, posee propiedades interesantes como biocarburante con respecto al bioetanol como, por ejemplo, un mayor poder calorífico o una menor higroscopicidad, implicando una más sencilla adaptación a infraestructuras existentes. Por otro lado, el butanol es una "molécula plataforma" de gran interés capaz de reemplazar parcialmente las bases tradicionales obtenidas en petroquímica. Muestra de esto último es que la producción biológica de butanol, por ejemplo, no implica la utilización de propileno (materia prima del proceso de producción convencional). Por otro lado, la deshidratación de butanol en butadieno supondría un alivio en las tensiones comerciales existentes sobre esta olefina.

La fermentación ABE es llevada a cabo por microorganismos estrictamente anaerobios del género Clostridia, y comporta un metabolismo bifásico (acidogénesis y solventogénesis), acompañado de un ciclo celular complejo. A pesar del interés que suscita, la reindustrialización de este proceso se ve frenada por unos costes de producción importantes, que derivan de una elevada inhibición por producto y de un rendimiento intrínseco limitado (con varios co-productos compitiendo por la fuente de carbono). El primer punto implica una muy baja concentración de producto en el caldo final de fermentación. Esto acarrea

consecuencias negativas, como son los altos costes energéticos de separación o las cargas de sustrato muy diluidas (aumentando los volúmenes acuosos circulantes). Para que la fermentación ABE pueda volver a entrar en el mercado, se hace necesaria la intensificación del proceso, que permita optimizar la producción reduciendo al máximo el coste de ésta. De las diferentes estrategias de mejora que guían las investigaciones más recientes, las técnicas de recuperación in situ buscan paliar el problema de la toxicidad del producto por medio de una separación primaria integrada y concomitante a la fermentación. Según el agente de separación utilizado, se han estudiado varias técnicas, desde la adsorción (sólido-líquido) hasta el gas-stripping (gas-líquido). Concretamente, este trabajo trata la aplicación de una extracción liquido-liquido en el seno del biorreactor (fermentación extractiva)

El principal objetivo de la presente tesis es la mejora de la fermentación Acetona-Butanol-Etanol (ABE) extractiva, aplicando enfoques basados en el uso de disolventes específicos.

Se presenta una metodología detallada que permite integrar, en la evaluación del proceso de fermentación extractiva, la comprensión de los fenómenos físicos y biológicos, haciendo hincapié en las interacciones entre el disolvente utilizado y el metabolismo del microorganismo. En este sentido, el contenido de esta memoria se divide en dos bloques complementarios: un primer bloque, orientado a mejorar la identificación y comprensión de los fenómenos asociados a la fermentación extractiva, y un segundo, consistente en una evaluación global del sistema integrado mediante una visión orientada al proceso. Así, tras la selección global de disolventes presentada en el **Capítulo 2**, el resultado principal del **Capítulo 3** y del **Capítulo 4** es la mejora del conocimiento de la fermentación ABE extractiva. Para lograrlo, se investigó la cinética del producto y la dinámica de la población microbiana en configuraciones extractivas, con disolventes de naturaleza opuesta. En el **Capítulo 5** se desarrolló un modelo de fermentación ABE, con el fin de traducir los conocimientos adquiridos previamente en expresiones matemáticas. En el segundo bloque temático, se realizó una evaluación comparativa tecno-económica (**Capítulo 6**) en base a los resultados experimentales obtenidos en el primer bloque. El objetivo es analizar prospectivamente la distribución de costos principales e identificar cuellos de botella. Finalmente, el uso de un hidrolizado de celulosa procedente del proceso *organosolv* se evalúa como sustrato en el **Capítulo 7**. Esta última parte está orientada a desarrollar alternativas a la producción de ABE sostenible dentro de una biorrefinería 2G.

En el **Capítulo 2** se lleva a cabo una selección global multicriterio permitiendo clasificar las diferentes familias de disolventes según sus propiedades físicas para la separación (coeficiente de reparto, selectividad) y la respuesta biológica generada

en el microorganismo (biocompatibilidad, biodisponibilidad). Se concluye que el disolvente óptimo será fruto de compromisos entre criterios. Así, el coeficiente de reparto del butanol (que fija la cantidad de disolvente necesaria) es inversamente proporcional a la selectividad (que determina la cantidad de agua que será retirada con los productos, afectando a la etapa de regeneración). A su vez, el coeficiente de reparto disminuye cuando la biocompatibilidad del disolvente aumenta. De acuerdo con trabajos previos, en el **Capítulo 2** se identifica un comportamiento asintótico en la representación de la actividad metabólica en una fermentación ABE extractiva frente a la biocompatibilidad del disolvente empleado (expresada en términos del logP). La región asintótica se corresponde, para *C. acetobutylicum*, con valores de logP de entre 4 y 6, y engloba aquellos disolventes con la mejor capacidad de extracción, pero que pueden presentar cierta toxicidad o comportamientos inconsistentes en experimentos repetido. En el **Capítulo 2** se identifican dos alternativas opuestas potencialmente interesantes: un aceite vegetal altamente biocompatible (aceite de orujo), pero con un bajo coeficiente de reparto para el butanol (< 1 g/g), y un alcohol β-ramificado de tipo Guerbet (2-butil-1-octanol), que no había sido previamente evaluado para fermentación ABE extractiva. Este disolvente, que cae de pleno en la región crítica, presenta el mejor coeficiente de reparto de los disolventes evaluados (6.76 g/g) y la tercera mejor selectividad (644). Además, sorprendentemente, el rendimiento en butanol se aumentó en casi 30% con respecto a la fermentación control.

En el **Capítulo 3,** los disolventes previamente seleccionados (aceite vegetal, 2-butil-1-octanol) fueron evaluados a través de las cinéticas de fermentaciones extractivas, en botellas estancas en modo *batch*. Los perfiles de concentración en ambas fases del sustrato (glucosa), productos intermedios (ácidos) y finales (ABE) fueron analizados y comparados a los de una fermentación convencional. La recuperación concomitante de los productos inhibidores durante la fermentación conlleva el consumo total de la glucosa con ambos disolventes, mientras solo el 70% del sustrato es consumido en la fermentación control. La producción total de butanol en las fermentaciones extractivas excede la del control en 60 y 100% respectivamente con el aceite vegetal y el 2-butil-1-octanol. Además, se observó un aumento de rendimiento en butanol (46%) con el disolvente orgánico confirmando los resultados del **Capítulo 2**. La producción selectiva de butanol se produce en detrimento de la acetona (la ratio butanol/acetona se duplica en la fermentación extractiva con 2B1O). En la red metabólica de *Clostridium acetobutylicum* la acetona se produce de forma irreversible por descarboxilación a partir de acetoacetato, y su formación está ligada, por una actividad catalítica común (CoA transferasa), al consumo de ácidos (butírico, acético). Así, por equivalencia molar, se estima el porcentaje relativo de butanol que procede del consumo de ácido, y éste es hasta dos veces menor en presencia de 2-butil-1-octanol (en favor del

butanol producido por vía directa, más interesante desde el punto de vista del rendimiento). En contrapartida, una fase de adaptación hasta dos veces más larga se aprecia en las fermentaciones extractivas con 2B1O. Los resultados obtenidos en el **Capítulo 3** demuestran que las diferencias entre disolventes de extracción van más allá de lo esperado por termodinámica, y la actividad metabólica del cultivo parece estar significativamente influenciada por la presencia de 2-butil-1-octanol.

La investigación de los efectos de la fermentación ABE extractiva sobre la población microbiana es el principal objetivo del **Capítulo 4**. La bacteria solventogénica *Clostridium acetobutylicum* posee un ciclo celular complejo durante la producción de butanol. En modo *batch*, las células pasan por una primera fase vegetativa (bacilos, gran movilidad) que se suele asociar al crecimiento microbiano y formación de ácidos, para luego evolucionar hacia fenotipos de mayor tamaño y complejidad interna (formas "clostridia"), que preceden la esporulación. Las esporas libres, inactivas y resistentes, pueden volver a germinar si las condiciones ambientales son adecuadas. Definir el rol metabólico de cada una de las fases del microorganismo ha motivado la aplicación de diferentes técnicas analíticas capaces de monitorizar cualitativamente el paso de la fase acidogénica a solventogénica (como el seguimiento de la composición del gas de fermentación, el consumo de base/ácido o la polarizabilidad del medio) o de caracterizar en detalle la composición de la célula (Raman, espectroscopía de infrarrojos). Estos últimos métodos, aunque proporcionan una amplia información, requieren mucho tiempo de análisis y preparación. La citometría de flujo, técnica emergente y robusta aplicada en varios ámbitos de la biotecnología industrial, permite cuantificar la evolución de la morfología y composición de un cultivo bacteriano a partir de una sencilla preparación de la muestra. En el **Capítulo 4** se aplica la citometría de flujo para cuantificar la dinámica de las poblaciones microbianas de *Clostridium acetobutylicum* en modo *fed-batch,* en reactor de tanque agitado, con y sin disolvente presente *in situ*. Las subpoblaciones microbianas se clasifican en cuatro grupos en base a análisis complementarios de microscopía y a estudios previos de la literatura. En el caso convencional (sin extracción), el porcentaje de esporas aumenta de forma continua, pasado el shift metabólico, hasta alcanzar más de 20% al final de la fermentación. En esta fermentación, la concentración de butanol evoluciona de forma sincronizada a la familia de células vegetativas, en acuerdo con un estudio previo de la literatura. Este resultado corrobora que la célula vegetativa sea responsable de la producción de butanol en la fermentación ABE, y aporta una visión del ciclo celular diferente a la más comúnmente aceptada en la literatura. Además, en la fermentación extractiva con aceite vegetal, se observa que la producción de butanol se lleva a cabo predominantemente por células vegetativas, con muy baja actividad de esporulación. Por el contrario, con el

disolvente más polar y capacitivo, 2-butil-1-octanol, la formación de esporas se anticipa y alcanza más de 40% al final de la fermentación, a pesar de que la concentración acuosa de butanol se mantenga inferior a los niveles de inhibición.

Parte de la información obtenida en el **Capítulo 4** sobre la evolución cuantitativa de las distintas subpoblaciones microbianas que coexisten en el biorreactor, sienta las bases del trabajo de modelización realizado en esta tesis, y recogido en el **Capítulo 5**.

Varios trabajos de modelado de la fermentación ABE se recogen en la literatura, todos ellos construidos bajo hipótesis específicas y con grados de complejidad variables. Aunque la coexistencia en el biorreactor de varios fenotipos con actividad metabólica definida sea una evidencia, la heterogeneidad en el cultivo de Clostridia ha sido por mucho tiempo obviada en los trabajos de modelado ABE. Tomando como punto de partida los datos obtenidos en citometría de flujo, en el **Capítulo 5**, se desarrolló un modelo cinético de la fermentación ABE bajo la hipótesis de la existencia de un fenotipo solventogénico que posee crecimiento vegetativo. El ciclo celular propuesto gobierna la productividad en cada tiempo según las condiciones del medio (pH, composición). Concretamente, el shift metabólico (entre fases acidogénica y solventogénica) es inducido en el modelo por la transformación parcial entre las familias vegetativas acidogénicas y solventogénicas. De la misma forma, la formación de esporas responde en la simulación a la evolución progresiva de la familia solventogénica hacia formas esporulantes bajo una situación de estrés (en el modelo, una concentración de butanol crítica). El modelo propuesto describe de forma adecuada, la producción y consumo de los metabolitos extracelulares en fermentaciones batch sin control de pH (datos experimentales propios) y bajo diferentes niveles de pH impuestos, incluido pH 6, que deriva en una fermentación acidogénica no convencional (datos de la literatura). Además, el modelo proporciona información adicional acerca de la dinámica de las diferentes familias que componen la biomasa, y los datos simulados reproducen los datos obtenidos en el **Capítulo 4** por citometría de flujo para una fermentación estándar. Sin embargo, aplicado a la fermentación extractiva, considerando los datos de repartición termodinámica entre fases obtenidos previamente (**Capítulo 2**), el modelo sobreestima sistemáticamente la productividad de todas las reacciones consideradas. Las diferencias son particularmente importantes en el caso de la fermentación extractiva con 2-butil-1-octanol como disolvente. La simulación confirma que, más allá de la transferencia física de metabolitos entre fases (y, consecuentemente, reducción de la inhibición por producto), estén presentes fenómenos complejos de interacción en el metabolismo ABE, ligados a la toxicidad de los disolventes o a la presencia de la interfase, y deban ser considerados en futuras investigaciones.

En capítulos anteriores se cuantifica el aumento de la concentración del producto en la fermentación ABE mediante la integración de la extracción líquido-líquido in situ. El rendimiento de butanol también se incrementó en presencia de disolventes orgánicos específicos, como el 2-butil-1-octanol. El propósito del **Capítulo 6** es cuantificar la repercusión económica de los parámetros de fermentación obtenidos en el proceso general. Para ello, se realizan simulaciones con Excel 2013 y ASPEN Plus v7.3, para una planta de producción de butanol de 35 kt/año de capacidad como base de cálculo, tomando como input los datos experimentales. Se comparan cuatro configuraciones de fermentación extractiva diferentes, bien por la naturaleza del disolvente (2-butil-1-octanol o aceite vegetal), bien por el modo de operación (*batch* versus *fed-batch*). La demanda total de energía disminuye en todos los escenarios con respecto a la fermentación control, siendo la fermentación con aceite vegetal en modo *fed-batch* la que resulta en un mayor ahorro de energía (61%). Sin embargo, la rentabilidad mayor se obtiene con el 2-butyl-1-octanol en modo *batch*, reduciendo el precio de venta mínimo de butanol en un 29% sobre el caso base. Para este escenario, los resultados numéricos indican más del 80% de reducción de aguas residuales a tratar, junto con un 34% de ahorro en materias primas. Además, un análisis de sensibilidad del precio de la fuente de carbono (parámetro más sensible y que gobierna el costo global en todos los casos) demuestra que la rentabilidad relativa de los casos podría verse alterada. Así, se encuentra que por debajo de un límite de precio del azúcar (aproximadamente 200 €/t), el uso de aceite vegetal como disolvente de extracción se vuelve más interesante.

Del **Capítulo 6** se concluye que el precio de la fuente de carbono es el parámetro que gobierna la rentabilidad en todas las configuraciones. La búsqueda de materias primas sostenibles, abundantes y de bajo coste es uno de los pilares imprescindibles para acelerar el desarrollo de este tipo de procesos biotecnológicos. Para incorporar el proceso integrado de fermentación ABE extractiva en el concepto de biorrefinería, se validó, en el **Capítulo 7**, el uso como fuente de carbono de un hidrolizado de celulosa de madera de haya, procedente del proceso *organosolv*. Para la realización de este trabajo se utilizó la cepa *Clostridium beijerinckii CECT 508*, que, en estudios precedentes, ha resultado minimizar la represión al consumo de xilosa (en presencia de glucosa) con respecto a la cepa habitual. En una primera parte, la fracción de celulosa hidrolizada es fermentada y comparada con el medio sintético en procesos de fermentación extractiva (con 2-butil-1-octanol) y convencional. La producción total de ABE aumenta en 40% con el hidrolizado de celulosa en comparación con la fermentación de control convencional y se incrementan un 10% con la fermentación extractiva. Sin embargo, la fase de adaptación (*lag time*) en las fermentaciones con hidrolizado se duplica, probablemente debido a la presencia de inhibidores remanentes del

pretratamiento *organosolv.* Para intentar paliar este retraso, en una segunda parte, se aplica una etapa de desintoxicación enzimática con lacasa previa a la fermentación. El objetivo es transformar los fenoles libres del medio en moléculas de mayor peso molecular y potencialmente menos tóxicas. Esto se realiza dentro de dos posibles escenarios industriales: con y sin operación unitaria de separación sólido-líquido tras la hidrólisis enzimática. La solventogénesis es completamente efectiva en todas las configuraciones, pero los beneficios del tratamiento enzimático con lacasa se revelan únicamente cuando los sólidos remanentes de la hidrólisis están presentes en el medio. En este caso, se observa una mejora del 25% en la producción final de ABE total. Con respecto a una implementación industrial futura, la fermentación directa después de la hidrólisis enzimática aliviaría el costo de la inversión y simplificaría el esquema del proceso

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1. Introduction

OUTLINE

1.1 Towards a biobased economy: the role of white biotechnology

We live in a finite world facing complex and global challenges.

World appetite for energy, chemicals, products and food is continuously growing. In 2016, we consumed 160000 TWh of primary energy, which is 25 times the 1800's consumption (OurWorldInData, 2018). This never flattening trend is driven by population growth. Two hundred years ago, less than one billion humans were living on Earth. Today, predictions announce an increase from seven to more than nine billion people by 2050 (United Nations, 2017). Moreover, the world is getting wealthier. The transition from poor to middle class brings evident benefits to population quality of life, but it also brings resources intensive habits like the use of individual car, electronic devices, increased share of meat in the nutrition mix, etc.

On the other hand, fossil fuels are the corner stone of this growth that took off during the industrial revolution starting with coal, followed by oil and more recently driven by gas. Oil is an impressive energy and material vector: liquid, cheap and abundant during many years. It progressively replaced all traditional alternatives, creating an absolute dependency not only related to energy but also to derived materials.

Till the beginning of the 2000's, concerns were focused on the ending of fossil resources dealing with scarcity, high prices and geopolitics (Landeweerd et al. 2011). High prices led to technological progress that unlocked massive quantities of oil and gas (e.g. fracking, offshore, deep sea), overcoming mid-term concerns on resources availability, even if spot prices remain subjects to the Middle East mood. Nonetheless, higher concerns are emerging regarding fossil resources, and they are more related to the excess rather than the scarcity of carbonized material.

Indeed, global climate change and its catastrophic consequences are one of the world's most pressing environmental concerns. Since the start of the twenty-first century, the annual global temperature record has been broken five times (Lindsey and Dahlman 2018). To face this worrisome problem, the whole production scheme should be rethought to minimize emissions of greenhouse gases, especially carbon dioxide. This implies a reduction in the use of fossil fuels which are believed to be a major cause of global warming, accounting for around 60% of CO_2 production (Emissions Gap Report, 2018). No less important, other environmental challenges must to tackle nowadays, like air and water pollution or cumulus of (non-biodegradable) wastes, especially plastic. The increased demand of "green" products, i.e. products with a lower carbon footprint, is a reality.

The perception that the end of the fossil era is already here is certainly more present than ever and the research for an alternative sustainable model urge. This implies the transition towards a bio-based economy, with an increased use of bio-based products. While energy production can be achieved from a large panel of renewable sources (e.g. wind, sun, water, etc), alternatives for the generation of chemicals, materials, or liquid fuels, are almost limited to biomass conversion. Theoretically, the advantages are clear, as can be seen in Figure 1.1: CO_2 closed circle, renewability and availability of resources. But, how to succeed this transition in a sustainable way to benefit all parties: preserve the planet while fulfilling human needs?

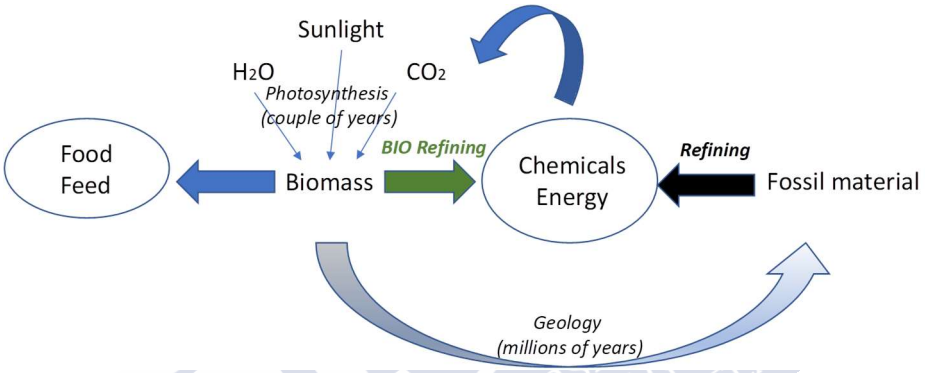


Figure 1.1. Cycle of biomass conversion

The use of biomass in industry is not new. Some examples are the use of wood as an energy carrier since the discovery of fire, or the development of the first automobile engine running on bioethanol in 1860. The use of biofuels declined in the first part of the 20th century, when crude oil and coal exploration began all around the world, favoring the development of an increasing petro-based industry (Landeweerd et al. 2011).

The transition (or return) to a biobased system is a challenging task due to conflicting global interests. Biomass, even if renewable, is limited, and thus a certain hierarchy in its uses should be established. As an example, the first generation (1G) biofuels (those derived from edible biomass) industry was accelerated by the establishment of energy policies and directives. For example, one of these directives is the EU's binding requirement of 10% of biofuels blending in the transportation (Directive 2009/28/EC9 of the European Parliament). But this turned out to have many adverse effects in terms of shifts of land (and water) use all over the world, and especially in developing countries (Gasparatos et al. 2013). So that, biodiesel from palm oil may have been responsible for up to 6.5% of direct deforestation in Malaysia (Gao et al. 2010). On the contrary, it has also been reported that the land foot print of 1G ethanol would be negligible (Rulli et al.

2015). On the top of that, the "food versus fuel" debate remains open: is it conceivable to produce biomass for biofuel production while there is already much pressure on land and water to produce enough food for the increasing population? (Pearce 2005, Monbiot 2004). This eventually resulted in a boost in the development of 2nd (2G) or even 3rd (3G) generation biofuels, using lignocellulosic material and marine algae as substrate, respectively, which will compete less with nutrition and could partially solve some of the social and environmentally concerns raised by 1G biofuels (Mohr and Raman 2013, Gao et al. 2010). First commercial-scale 2G ethanol industrial plant started in 2013 and, since then, eight more have opened all around the world (most of them in the US, two in Brazil and one in Europe), in all cases with existing mature 1G industry and policy incentives supporting cellulosic ethanol (Alfano et al. 2016).

On the other hand, with the introduction of new bulk biochemicals as building blocks, new processes and technologies will need to be developed. Therefore, substantial investments would be required, creating new opportunities. In certain cases, it might be interesting to partly benefit from "old transformation processes", inserting the new biochemical block in a classical schema (Figure 1.2). An already existing industrial example is the production of polymer grade by dehydration of bio alcohol, which replaces petro-based alkene as bulk chemical. This allows the synergic utilization of existing downstream polymerization units and the new biotechnology industry (Bio-based News, 2019).

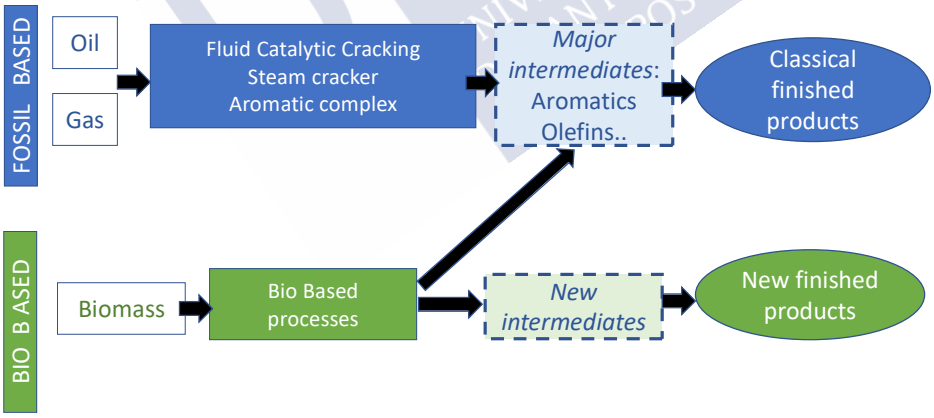


Figure 1.2. Bulk chemical production: integration into traditional scheme (adapted from IFP Energies Nouvelles, 2014)

The development and definition of the future generation of biorefineries, and more specifically the lignocellulosic biorefinery, as integrated systems to convert biomass and organic wastes into different products (chemicals, fuels, materials, energy) is key in the transition towards a biobased economy (Kamm et al. 2007). In

this context, the “white biotechnology”, defined as the modern use and application of biotechnology for the sustainable production of chemicals, materials and fuels (EuropaBio, 2017), might also play an essential role.

Fermentation processes have been used by mankind for thousands of years. Its history dates to at least 7000 BC, when microorganisms were first used in the homemade production of cheese. However, it last until the second part of 19th century that biotechnology was implemented at industrial level (ethanol, acetic acid, lactic acid and citric acid, etc). The advances in technology, a much deeper understanding of cell metabolism and the development of new genetic tools enforce the key position of white biotechnology in the transition to a biobased economy. It is expected that white biotechnology could positively impact the so called “3P dimensions”: planet, profit and people (EuropaBio, 2015). The planet benefits, since biotechnological processes use efficiently renewable raw materials and create little end-of-pipe waste (often biodegradable). It creates profit, since economy benefits of the introduction of more efficient and less energy-intensive processes. Finally, this will enable the creation of new jobs and new players in the market. Industrial biotechnology is expected to contribute above one million jobs to the EU economy by 2030 (EuropaBio, 2015).

Finally, the transition to renewable raw materials for energy and products, and the replacement of oil-based process by new biotechnology routes, might not be enough to solve the global human resource equation. Indeed, reducing the negative impact of the so-called linear economy (make, take, dispose) is necessary, and rethinking the production model with the principles of circular economy is necessary to guarantee long term sustainability. Therefore, innovation in biorefinery design should be included in a global innovation system that also refers to consumer behavior, product use and waste management (European Environment Agency, 2018).

1.2 Biobutanol

Butanol is a four-carbon alcohol (C_4H_9OH) with four structural isomers: n-butanol (1-butanol), iso butanol (2-methylpropan-1-ol), sec butanol (2-butanol) and tert butanol (2-methylpropan-2-ol). The former (n-butanol) is the most important commercial isomer. The term biobutanol refers to butanol made from biomass feedstocks. Biobutanol is both an important bulk chemical with a wide range of industrial usages and a high potential alternative biofuel.

1.2.1 Biobutanol, an advanced biofuel

Total world consumption of energy for transport has been reported to be around 2Gtep/y, and liquid biocarburants count for 3.7%, with an increasing trend in their consumption (boost of 5% between 2015 and 2016, while the overall demand for transport carburant grew only 1.6 %) (IFP Energies Nouvelles 2014). Ethanol manufactured via yeast-based fermentation of corn or sugarcane is the main substitute for the gasoline pool, and addition of 10% ethanol to gasoline in several European countries has become very common. (Biethanolcarburant, 2019).

For the last three decades biobutanol has been presented as an advanced biofuel for gasoline replacement. Indeed, it presents some advantages over ethanol (Durre 2008, Zheng et al. 2015, Wang et al. 2017): a) higher energy density, b) lower heat of vaporization and lower Reid vapor pressure, which means lower volatility and vapor emissions, c) less hygroscopic (butanol is only partially miscible with water), which implies not only a lower corrosion risk but also better adaptability for distribution through existing gasoline pipelines systems. Table 1.1 summarizes some relevant properties of butanol, ethanol and gasoline. Unlike ethanol, butanol is often considered as a “drop in” biofuel, meaning that it should be equivalent for end use as petroleum derived fuels (Karatzos et al. 2014). Butanol can be blended with gasoline in a higher proportion than ethanol, as the required air/fuel ratio and energy content are more like that of gasoline. Biobutanol blends up to 16% by volume are currently allowed in the USA as a legal fuel equivalent to E10 (Alternative Fuels Data Center, 2019).

Table 1.1. Comparison of physic and chemical parameters of ethanol and n-butanol over gasoline (data from Lee et al. 2008, Kujawska et al. 2015, Cascone 2008).

	Gasoline	Ethanol	n-Butanol
Energy density (MJ L^{-1})	32	19,6	29,2
Vaporization heat (MJ kg^{-1})	0,36	0,9	0,43
Motor Octane Number (MON)	81-89	102	78
Boiling point, ºC	27-225	78-78,4	117-118
Air-fuel ratio	14,6	9	11,2
Density at 20ºC (g ml -1)	0,74-0,8	0,785	0,81
Water solubility at 25 ºC (%)	9,1	100	<0,01

Some of the most important disadvantages of butanol with respect to ethanol are: a higher viscosity, lower octane number and higher toxicity to humans and animals. Furthermore, butanol does not take advantage of decades of experience in its use as a fuel (blendstock and primary fuel), as in the case of ethanol. In fact, biobutanol production is currently more expensive than bioethanol which has longtime hampered its commercialization. Despite its potential interesting properties, it is not yet certain whether it will be accepted by the transportation industry as the main alternative fuel. It will probable depend on the results from emerging investigations of novel and more economic alternatives to traditional ABE fermentation. The short-term forecast for biobutanol as a fuel is limited, and production data are scarce in Europe. In the USA, it has been reported that 12000 gallons entered the market in 2013, none in 2014 and 2015 and more than 125000 gallons in 2016 (Alternative Fuels Data Center, 2019).

1.2.2 Biobutanol, a bulk chemical

Currently, butanol is basically produced from petroleum to be used as a solvent and a chemical intermediate to be further transformed into many important products. As a solvent, it is used in the manufacture of paints, dyes, coatings, varnishes, resins, etc. As a chemical intermediate, most of current butanol worldwide production is transformed into methacrylate esters, butyl acrylate, butyl acetate, dibutyl phthalate and sebacate (Durre 2008). Further transformation of

these molecules leads to various plastics and polymers. Other final products from butanol include cleaners, flotation agents, cosmetics, as well as the production of antibiotics, hormones, and vitamins (Hestekin et al. 2013).

In 2018 the worldwide chemical demand for n-butanol was approximately 5 million tons (Europe accounting for 24% of the supply), and the average growth is expected to be 4.5% per year from 2019 to 2024 (Axiom. Market Research and Consulting ,2018).

Most butanol produced today is synthetically derived from propylene (by the oxo process), thus, its production cost is directly linked to petrochemistry industry trends. The propylene production in Europe is extremely sensitive to the price of naphtha, which represents 80% of the steam cracking feedstocks. But the development of shale gas in USA has progressively forced the replacement of naphtha by ethane as steam cracking feedstocks. Since the olefin yield structure at the outlet of the steam cracker depends on the feedstock, the global production of propylene has been reduced by a factor of 20 using ethane instead of naphtha (IFP Energies Nouvelles 2014). This creates a commercial tension on this C3-olefin, which could negatively impact the production cost of butanol by the oxo process.

Other heavy olefins are also impacted by the evolution of the petrochemical industry. It is the case of butadiene, whose production is reduced by six with the switch in cracker feedstock, while demand is still growing, leading to a possible shortage of not only butadiene, but other derived C4-based platform chemicals, such as butanediol. Butanol dehydration to butadiene is an alternative pathway that can alleviate the tension on this intermediate (Kruger et al. 2018).

The increasing tensions surrounding the availability and price of some key petrochemical intermediates lead to a true opportunity to develop a new bulk biochemical industry, and concretely to expand biobutanol production capacity. Mariano et al. (2013) evaluated the economics of greenfield projects of 1G biorefinery by co-producing ethanol and butanol from sugarcane juice in Brazil. They concluded that the investment for butanol production would be attractive only if butanol is sold as a building block in the chemical market. Furthermore, the intrinsic low butanol mass yield in conventional ABE fermentation could represent an important hurdle for its penetration in the fuel market.

1.3 Brief history of ABE fermentation

The biochemical production of acetone and butanol was one of the first large-scale industrial fermentation processes. It ranked second in importance to yeast ethanol fermentation, during the first part of the 20th century. The main reason for

its almost total cessation in the 1960's was its non-ability to compete economically with the petrochemical process.

In 1862, butanol was biologically synthetized for the first time by Louis Pasteur, who had isolated an anaerobic butyric-acid-forming bacterium (*Vibrition butyrique*). Some years later (1876), Albert Fitz reported bacterial glycerol fermentation into butanol and butyrate by *Bacillus butylicus*. In 1893, Martinus Beijerinck isolated two butanol-forming bacteria: *Granulobacter saccharobutyricum* and *G. butylicus* (Durre 2008). But it was at the beginning of 20th century, when the Russian chemist Chaim Weizmann (former Israeli President) identified the most investigated butanol-producer strain, *Clostridium acetobutylicum*, which produced large amounts of acetone and butanol from starch. These were the first results concerning the ABE process development and were part of a patent presented in 1915 (Jones and Woods 1986).

The Weizmann process played an important role during the World War I: the bacterial fermentation allowed producing large quantities of acetone, which was used in the manufacture of gunpowder cordite. Before the war, acetone was produced chemically from calcium acetate, but its importation in England during the war was limited. Because of the strategic need for large volumes of acetone, some facilities based on the Weizmann process were built in the UK using maize starch as substrate. However, German blockage affected the supply of grain, and rationing was soon introduced, so the English government could not release more starch for solvent production. After a failed attempt to run fermentation from horse chestnuts, the fermentation process was transferred to Canada (Ranjan and Moholkar 2012). Some acetone was also produced in France and in India, but the largest scale plants were built in Canada and in the USA. At that time, butanol was an unnecessary by product in the ABE process and had no commercial value (Zverlov et al. 2006).

After 1918, huge quantity of acetone was no longer required, and the biological process was about to be abandoned. However, at this time the automobile industry was expanding quickly and, few years later, started demanding large quantities of butyl-acetate ester (derived from butanol) as a solvent for car lacquers. Solvent production was restarted, and new fermentation plants were built. Butanol also found an application in the synthetic rubber industry (Durre 2008). In 1923, a major problem developed during fermentation operation -later diagnosed as a bacteriophage infection- forced to stop the 52 fermenters of the ABE plant in Terre Haute (Indiana, USA). At that moment, the butanol demand was so highly increasing that an entirely new plant was built in another state while the bacterial infection problem was investigated. Between 1925 and 1927 the two existing plants

in the USA expanded rapidly: 100 tons per day of solvent were produced (Jones and Woods 1986).

At the beginning of the 30's, the synthetic production of acetone from petroleum first appeared as a threat for the availability of the ABE fermentation, and much research were done on the isolation of strains which could ferment higher concentrations of starch or other substrates. By 1935, the isolation of new strains which were able to ferment up to 6.5% sugar in molasses allowed increasing plant capacities by 60% (Dürre 2011). After 1936 (when the Weizmann patent expired), new facilities were built in a number of countries including Japan, India, Australia, South Africa, Egypt, Brazil and the USSR (Jones and Woods 1986).

During World War II, the demand for acetone again rose to a very high level. Due to the problems of importing molasses into Britain, large amount of acetone was imported from the USA, where fermentation plants where functioning at full capacity.

In 1945, 66% of the total butanol and 10% of the total acetone worldwide production came from the ABE fermentation, becoming the largest scale biotechnological process ever run second to ethanol yeast fermentation. After this moment, ABE fermentation gradually assumed less and less importance. The biotechnological route could not compete with butanol produced synthetically because of the availability of much cheaper petroleum feedstock and the increasing prices of the molasses (Zverlov et al. 2006, Jones and Woods 1986).

The fermentation process ceased in the USA and Europe in the 1960s while some production remained in South Africa until 1982. In the USSR, ABE production plants were active until the disintegration during the 90's (Zverlov et al. 2006), and in China, ABE production also completely ceased in the 90's (Ni and Sun 2009).

Nowadays, even if most of n-butanol is produced chemically from petroleum, feedstock prices instability and renewal interest in green chemistry resulted in the consideration of the ABE fermentation as an alternative for butanol production. After decades of stagnation, it can be reflected by the dramatic increase in ABE publications and an important number of new fermentation plants.

China has led efforts to re-commercialize the ABE fermentation in the last decades, with the construction of dozens of plants (Jiang et al. 2015, Ni and Sun 2009). From 2006, the total annual ABE production capacity from these plants reached 210000 tons. However, some projects expected to respond to a very high n-butanol demand in China have been retrofitted in the last five years due to the rapid drop in oil price (Xue et al. 2017) together with the recent interdiction of 1G feedstock for non-food products (Jiang et al. 2015). In Brazil, an industrial plant integrated in a 1G biorefinery is currently operated by HC Sucroquimica, with an

annual capacity of 8000 tons of ABE from sugarcane juice (Pinto Mariano et al. 2013). Moreover, SGBio Renewable, a joint venture of GranBio (Brazil) and Solvay (Belgium), plan to build a 2G biobutanol plant in Brazil, using as substrate bagasse and sugarcane straw (Biofuels Digest 2015). In the USA, recent ABE fermentation plants are retrofits of existing corn ethanol plants, and two leading companies (Gevo and Butamax) are investigating alternatives to traditional ABE fermentation. A first commercial scale of 18 MGPY cellulosic biobutanol plant from corn was started in 2012 by Gevo (Alternative Fuels Data Center 2019). In Europe, several academic and industrial actors are active in research for an intensified process. As an example, the ButaNext consortium is a multi-disciplinary team comprised of companies and research centers, whose aim is to optimize biobutanol production value chain: biomass pre-treatment, fermentation, downstream processing and blending. In this context, VITO (Belgium) has built a demonstration unit to test integrated (*in situ* recovery) techniques applied to ABE fermentation in the context of ButaNext H2020 project. (Kujawska et al. 2015)).

1.4 *Clostridium* genus bacteria: metabolism and cell cycle

The ABE fermentation is carried out by *Clostridium* sp. bacteria under strictly anaerobic conditions. The genus *Clostridium* is a heterogeneous collection of gram-positive, spore forming bacteria. Their optimal temperature is between 33 and 36 ºC, they are then classified as mesophilic. Several species of *Clostridium* are known to produce butanol including *C. acetobutylicum, C. beijerinckii, C. saccharoacetobutylicum, C. saccharoperbutyilacetonicum* or *C. pasteurianum*. Some Clostridia have been evaluated for isopropanol production, including strains of *C. beijerinckii* and *C. isopropylicum.* Clostridium genus bacteria are able to metabolize C5 and C6 sugars and can grow on polymeric substrates such as starch, but not on cellulose. Several enzymes produced by these bacteria, including amylases and glycosidases, allows to hydrolyze the polymeric substrates into monomers (Bankar et al. 2013, Jones and Woods 1986, Ezeji et al. 2007).

ABE fermentation is a complex process that involves two phases: acidogenic (growth associated production of carboxylic acids with a consequent decrease of pH) and solventogenic (assimilation of acids into solvents, during a pseudo stationary phase) (Lütke-Everlosh and Bahl 2011). Thus, the three main classes of products obtained during this process are solvents (ethanol, butanol, isopropanol or acetone), organic acids (acetic, lactic and butyric) and gases (CO_2, H_2).

1.4.1 Metabolic pathways

Classical biochemical pathways from glucose have been well established for Clostridia genre bacteria and are depicted in Figure 1.3. Glucose or xylose are

transformed into pyruvate by different metabolic ways. Glucose is metabolized by the Embden-Meyerhof-Parnas (EMP) pathway with the conversion of one mole of glucose to two moles of pyruvate. The energy released allows the net production of two moles of adenosine triphosphate (ATP) and two moles of reduced nicotinamide adenine dinucleotide (NADH) (Figure 1.3). Xylose is metabolized into pyruvate by pentose-phosphate pathway (PPP), where xylulose and fructose-6-phosphate are transformed into glyceraldehyde 3-phosphate, which enters the EMP pathway. In this case, three moles of xylose give five moles of pyruvate, five moles of ATP and five moles of NADH, leading to a lower energetic yield per mole of substrate than the former case.

After pyruvate formation, the microorganism enters the acidogenic phase, which is associated to the biomass exponential growth and leads to the production of butyrate, acetate, carbon dioxide and hydrogen, and ethanol and acetoin in small volumes. The oxidative conversion of pyruvate into acetyl coenzyme A (and CO_2) is catalyzed by pyruvate ferredoxin oxidoreductase enzyme, in the presence of coenzyme A (CoA), and is associated with hydrogen formation.

Acetate is synthesized from acetyl-coA by the action of phosphotransacetilase (pta) and acetate quinase (ack). Two molecules of acetyl-coA can also be condensed into acetoacetyl-coA by the action of thiolase enzyme (thl). The acetoacetyl-coA is in turn converted into 3-hydroxylbutyril-CoA, and butyril-CoA, from which butyrate is produced by the action of phosphotransbutyrilase (ptb) and butyrate kinase (buk). Butyric and acetic acid are produced during acidogenesis in an analogous step resulting in the production of acyl phosphate and correlated with energy generation in the form of ATP. Nevertheless, too large quantities of these acids in the broth seem to be toxic towards the bacteria, since pH gradient across the cell membrane is collapsed leading to partial inhibition of the metabolic functions (Jones and Woods 1986).

The onset of solvent production implies a switch in carbon flow from acid-producing to solvent-producing pathways (Figure 1.3). During this phase, solvents (ABE) are produced and the acids are also converted into these products, resulting in a typical 3:6:1 (A: B: E) molar ratio, concomitantly with the continued consumption of carbohydrate (Jones and Woods 1986). This phase leads to an increased pH in the broth, which allows preserving the metabolic activity of the cells. Considering this, the transition towards the solventogenesis has been postulated like an “adaptative response” or “detoxification process” of the microorganism against inhibitory effects of cumulative organic acids, which result in conditions unfavorable to growth (Bankar et al 2013, Lütke-Everlosh and Bahl 2011).

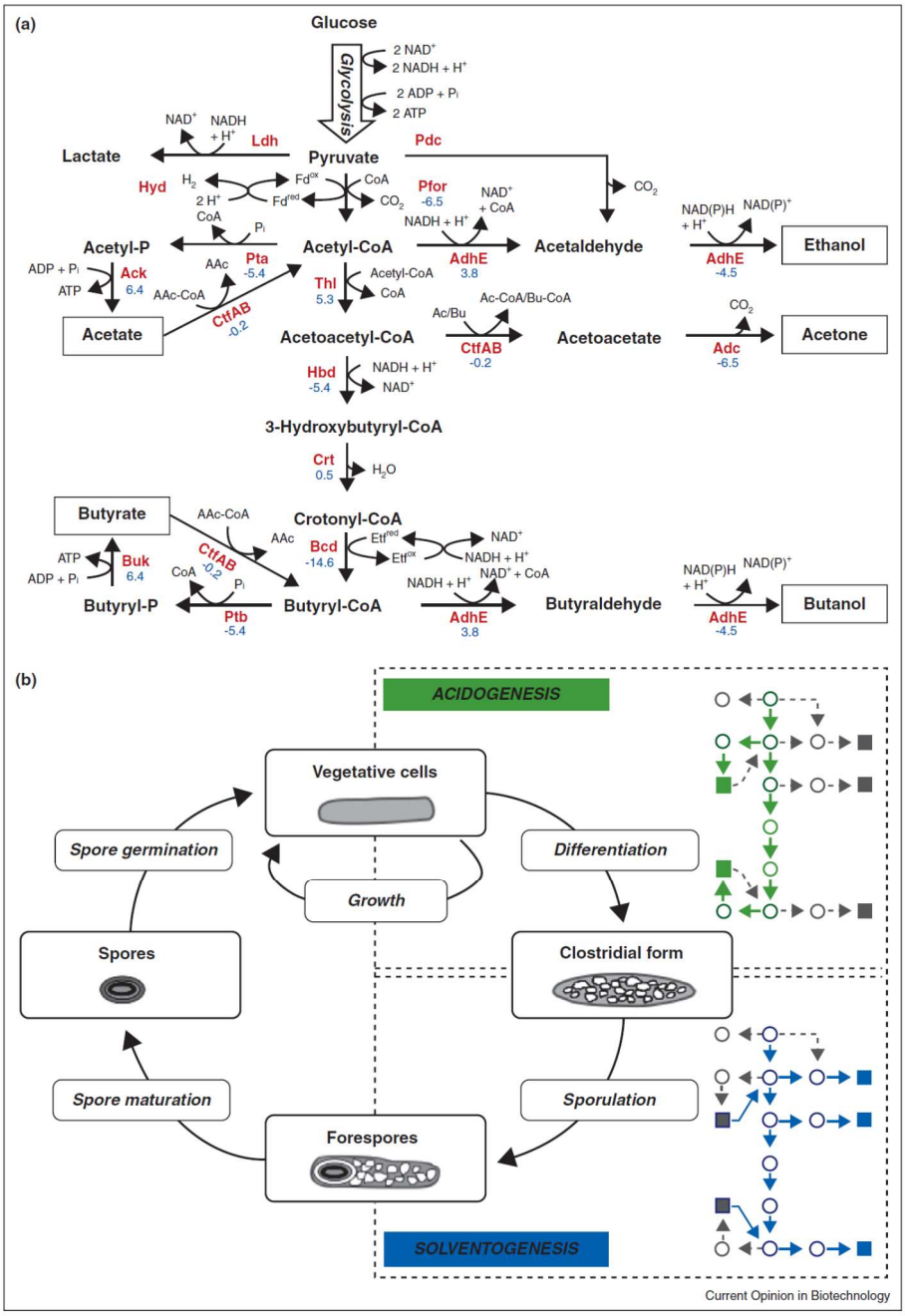


Figure 1.3 Metabolic pathways and cell cycle of Clostridium acetobutylicum (from (Lütke-Everlosh and Bahl, 2011))

Several enzymes are key in butanol synthesis. Some of them participate both in acid and solventogenic step, like thiolase (thl), 3-hydroxybutyril-CoA dehydrogenase (hbd) or crotonase (crt). The reduction of butyril-CoA to butanol is mediated by butyraldehyde dehydrogenase or butanol dehydrogenase (bdh). Even if metabolic activity of this phase differs between microorganisms, the uptake of both acids (acetate and butyrate) is usually correlated with the production of solvents (butanol and specially acetone), and numerous mechanisms have been postulated in the literature (Bankar et al. 2013, Jones and Woods 1986). Both acetate and butyrate can be utilized during the conversion of acetoacetyl-CoA into acetoacetate. The latest is then irreversibly decarboxylated to produce acetone. Thus, in a normal batch fermentation, the butanol yield would always be constraint by the acetone production during solventogenic phase. The solvents produced during the solventogenic step -and specially butanol- are also toxic to the bacteria cells, since they can modify or destroy their membranes. Consequently, a critical total ABE concentration exists, and it is reported to be approximately 2% (wt). (Jones and Woods 1986)

1.4.2 Cell cycle

Clostridia follow a complex cellular cycle during ABE fermentation (Figure 1.3). During acid production (exponential microbial growth), the so called “vegetative cells” are predominant. They are highly motile and rod shaped (bacillus). When solvent production begins, cells start to synthetize granulose as an internal storage compound and evolve into fattened cigar-shaped “clostridial cells”. Some of them will then sporulate, and the granulose is employed by the microorganism as energy and carbon source to achieve endospore formation (Lütke-Everlosh and Bahl 2011). Spore formation allows the bacteria to survive longtime under adverse conditions in terms of pH, O2, temperature etc. The free spores ejected into the broth have no metabolic activity, but this resistant form can germinate if environmental conditions become adequate to fermentation (Schuster et al. 1998). Therefore, distinct subpopulations of the microbial culture will coexist in the bioreactor during ABE fermentation, each one with specific phenotype and metabolic activity.

1.4.3 Factors triggering metabolic switch

The comprehension of the factors affecting the metabolic switch of Clostridia is key to start and maintain solvent production. External and internal pH, acid concentrations or nutriments concentration are variables that have been postulated to trigger the transition towards solventogenesis.

The pH is likely the most important factor in the balance of both fermentation phases. It has been reported that in cultures kept at a pH higher than 6, organic acid production is boosted and solventogenesis can be repressed (“acidogenic

fermentation" phenomena) (Maddox et al. 2000). Nevertheless, the optimal pH for solvent production depends on both the microorganism and the culture operating conditions. For *C. acetobutylicum* ATCC 824, the optimal pH range for solventogenesis is 5.5-4.3 (Jones and Woods 1986). pH is correlated with the accumulation of undissociated acids, which can penetrate the cell membrane and whose concentration is boosted while pH decreases.

Moreover, in batch or continuous cultures maintained at a pH higher than 5-6, solvent production was induced by supplementing the medium with butyrate and/or acetate (Jones and Woods 1986). Independently from the external pH of the medium, it was reported that undissociated butyric acid concentration of 0.5-0.8 g/L inhibits the growth of *Clostridium acetobutylicum* ATCC 824, while solventogenic phase is triggered with undissociated butyric acid concentrations from 1.5 to 1.9 g/L (Monot et al. 1984).

Other factor affecting the triggering of the solventogenic phase is the substrate or nutrients limitation. It has been stated that a severe carbon limitation induces low acid production, and consequently avoids triggering the solventogenic phase. The effect of other nutrients like ammonium or sulfate has also been studied. The failure to produce solvents in severe limitation of these nutrients is due to a too low generation of acids (Jones and Woods 1986). Nevertheless, it has been observed that limitation of certain nutrients like nitrogen (Baba et al. 2012) or phosphate (Bahl et al. 1982) may be advantageous for the onset and maintenance of solvent production. Recent studies revealed that the presence of calcium in the fermentation broth preserves the solventogenesis in those fermentation inhibited by formic acid (Qi et al. 2017).

1.5 Options to overcome the limitations of ABE fermentation

The interest for the research and development of biobutanol production has increased over the last decades. This is reflected in the presence of new players in the market and the increasing list of industrial projects. However, it is still necessary to overcome some major drawbacks to improve the competitiveness of the biological process and to make the large-scale production economically viable.

Six main process parameters can be considered to estimate the performance of the ABE fermentation: duration time or residence time (depending on batch or continuous operation mode), product concentration, volumetric productivity, product yield, product ratio (A: B:E) and feed concentration. These parameters (only four and independent) are responsible of the global performance and final cost of the biological butanol production. Several macroscopic techno-economic evaluations of the ABE fermentation have been reported in literature (Mariano et al. 2011, Qureshi et al. 2013). Even though the result strongly depends on the basis

assumptions (e.g. type and price of raw material, volume and cost of bioreactor, utilities, etc) the final repartition between CAPEX (CAPital EXpenditures), OPEX (OPerating EXpenses) and the cost of the raw materials will always be directly correlated with the above-mentioned parameters (Figure 1.4).

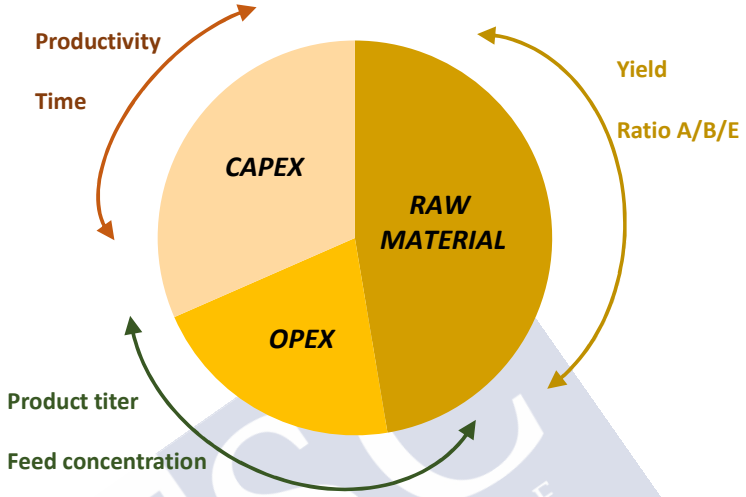


Figure 1.4. Distribution of the three main economic post in conventional ABE production process (repartition adapted from Gapes (2000))

The two main constraints of the ABE fermentation are:

1) *The strong end-product inhibition*. Clostridia have a limited tolerance to the fermentation products, which can dissolve in the cell membrane, disturbing its integrity and affecting its specific permeabilization (Jones and Woods 1986). Thus, the main products of ABE fermentation (butanol, acetone and ethanol) are known to be toxic to Clostridia. Besides, other co-products (organic acids, CO_2) present at lower concentrations, could also be growth inhibitors. As a result, even if the final products composition can vary depending on the strain used or operating conditions, the typical fermentation broth contains no more than 20 g/L of ABE (Dürre 2011, Green 2011, Ezeji et al. 2010, Oudshoorn et al. 2009). However, the final product concentration is not the only process parameter impacted by end-product inhibition. For example, batch process will stop when the inhibition threshold in the fermentation broth is attained, increasing the relative time dedicated to cleaning, filling and sterilization. Moreover, it can be expected that intrinsic kinetics of the process will be affected by the presence of inhibitors. Consequently, global volumetric productivity might also be reduced. The limited final product titer increases the recovery costs, but also imposes a maximal sugar

loading. Therefore, the water usage is increased, impacting the wastewater treatment cost.

2) *Low product yield related to Clostridia metabolism*. Butanol is one of the three main secondary metabolites of ABE fermentation. Moreover, solvent production is accompanied by an important CO_2 production, which represents a non-negligible carbon loss.

Table 1.2 compares the performance of both ABE fermentation and ethanol fermentation from yeast.

Table 1.2. Comparative performance of ABE and Ethanol fermentation

	ABE/Butanol	Ethanol
Yield (g product/g sugar)	0.34/0,17	0.48
Productivity (g product/l/h)	0.5/0,25	2-3
Concentration (g product/l)	20/10	100
Product ratio (molar)	A/B/E: 3/6/1	No co-product
Sugar concentration (g/L)	60	200
Batch time (h)	40	33

The three main research axes to overcome the drawbacks of the process are: a) microorganism, b) process development and intensification and c) research for renewable and cheaper substrates.

The first one deals with the modification of the microorganism by improving its tolerance towards the toxic products by gene regulation, thus enhancing the final alcohol titers, or boosting one or several metabolic pathways to improve the final product yield, by suppression or inactivation of specific enzymes (Ezeji et al. 2007). A variety of butanol-tolerant mutants have been developed in the last decades. However, the strains engineered or selected for enhanced solvent tolerance offer only slight improvement, and maximal titers of 20-24 g/L of ABE have been reported (Staggs and Nielsen 2015).

A complementary option is to work not on the microorganism itself but on its environment by rendering the optimal conditions for its metabolic activity. This concerns the process development and intensification and englobes every action

on process variables that aim at improving the fermentation performance. The implementation of in situ product removal (ISPR) techniques is an option to alleviate product inhibition and would contribute to overcome the maximal concentration without modifying the microorganism. The implementation of these techniques will not only improve the fermentation efficiency but would also alleviate the downstream energy requirements. Another approach is to increase cell density by using for example cell recycle or biofilm bioreactors. This would enhance the volumetric productivity by reducing the size and number of the bioreactors, which directly relates to the capital expenditures (CAPEX).

Finally, the search of cheaper carbon sources not competing with nutrition is also important. For example, it has been reported that feedstock contributes up to 60% of the overall solvent production cost in a corn starch conventional plant (Gapes 2000). Therefore, to significantly reduce the operating costs and improve sustainability of the process, transition towards cheaper and more sustainable feedstocks such as wastes, and agricultural residues will be necessary.

1.6 ISPR techniques

The traditional way of bioprocessing is “straightforward” (Figure 1.5a). In the fermenter the substrate is converted into the desired product up to the critical concentration (inhibition threshold) or its own depletion (in batch operation). Subsequently, the product is recovered from the fermentation broth in a separation and purification section, usually composed of a train of distillation towers. This is also called ‘end of pipe’ or downstream section, which operates in a continuous mode: batch fermentation runs are sequenced to furnish a continuous product stream (fermented broth) to the continuous downstream section.

This method of bioprocessing would work relatively well in ABE process if obtained butanol had a high enough final concentration. However, the recovery by distillation of the product from a very dilute fermentation broth (approximately 2% wt for ABE solvent) is energy intensive. In addition, binary mixtures of butanol and water form a hetero azeotrope at 55% wt of butanol in the liquid phase, and an additional distillation column is necessary for complete purification in downstream section (Leland 2008). The amount of energy required per unit of alcohol recovered decreases as the concentration of alcohol in the fermentation broth increases. It has been reported that the energy required for producing 99% wt butanol from an aqueous stream at 20% wt butanol (instead of approximatively 1,5% wt reached at the end of the fermentation) could decrease from more than 30 to 4 MJ/kg butanol (Leland 2008). This means that the primary separation of butanol could be an excellent opportunity for alternative technologies which are less energy expensive at low alcohol concentrations.

The main objective of the application of an integrated or in situ product recovery (ISPR) technique is the mitigation of product inhibition. The removal of the inhibitory product as the fermentation proceeds allows the generation of a more concentrated butanol stream which lowers the downstream energetic cost (since higher final concentration is attained) and reduces wastewater generation (since higher concentrated carbon source feedstock can be used).

Several approaches exist to perform the integrated product recovery system consisting of a fermentation step coupled with a primary product separation (Woodley et al. 2008). Two setups are distinguished:

- *In situ* recovery: the concentration step occurs inside the bioreactor, where the product is partially separated (Figure 1.5b). The alcohol-depleted fermentation broth never leaves the bioreactor.

- *In stream* recovery: fermentation and primary separation are carried out concomitantly but in two distinct vessels. This implies the continuous pumping of the fermentation broth though another column containing the selective phase or material, which allows separating a fraction of the product (Figure 1.5c). The alcohol-depleted stream is returned to the bioreactor.

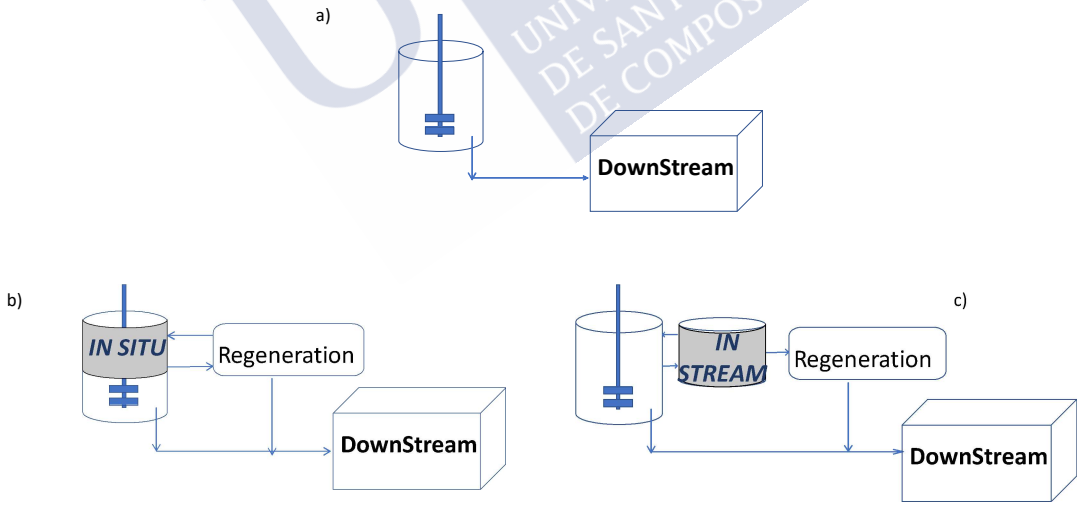


Figure 1.5. a) Straight forward bioprocess and b) In Situ, c) In Stream Recovery Techniques

Both recovery schemes eliminate the toxic effect by removing the compound from the fermentation broth: if the product is recovered as fast as it is produced, the inhibition is avoided, and the microbial culture could theoretically achieve higher productivity and product concentration. The advantages and disadvantages of both configurations depends on the type of separation (e.g. solvent extraction, adsorption, etc) and the operation mode (e.g. fed-batch, continuous) (Outram et al. 2017). *In situ* techniques usually mean an easier and more direct implementation, but also less modularity since the separation must totally respect the ongoing fermentation conditions. On the contrary, *in stream* recovery can benefit from specific separation conditions, at expenses of higher cost and complexity, together with increased contamination and cell damage risk.

ISPR techniques generally consist in "alleviating" methods. The aim is not to recover all the inhibitory product from the aqueous broth, but to partially remove the product to "alleviate" the inhibition. This has two consequences. First, a subsequent separation of the recovered product from the separation agent need to be considered in the design of an ISPR technique. For instance, a distillation (in the case of extraction solvent regeneration), or steam stripping step (if butanol is adsorbed on a solid). On the other hand, the downstream section must have been designed up to treat also the remaining product in the broth (which usually remains under the inhibition threshold). This can be achieved by a classical downstream section preceded by a mixing point (see Figure 1.5b, 1.5c), which would finally operate with at an intermediary level in terms of product concentration.

Apart from energy saving in the downstream section, and the use of more concentrated feedstock (which directly impacts OPEX), the integration of a primary separation in the bioreactor has also two important consequences leading to improve productivity (which directly impacts CAPEX):

- In the case of batch or fed-batch operations, the fermentation time at maximal productivity can be increased because the inhibition threshold is never reached. Thus, the turnover of reactors diminishes since the relative time between batches (filling, sterilization, cleaning, etc) is reduced compared to fermentation time.

- On the other hand, an ISPR technique boosts theoretically cell growth and product formation rates since inhibitory concentration level is decreased.

Moreover, it has been reported that yield could also benefit from ISPR from a biochemical point of view by relieving the stress on the microorganism. Removing the inhibitory compound would decrease the intracellular maintenance. Experimentally, enhancement up to 18% has been reported for ISPR coupled ABE

fermentation (Qureshi and Maddox 2005, Van Hecke et al. 2014). This would positively influence the raw material economic impact in process evaluation.

The application of various ISPR techniques on ABE process have been widely studied at laboratory scale, usually leading to improvements in productivity and toxicity reduction. Investigations about different ISPR techniques coupled to ABE fermentation have been the object of several research reviews (Outram et al. 2017, Abdehagh et al. 2014, Staggs and Nielsen 2015). The integrated product recovery of butanol from the aqueous broth can be based on the difference between physical and chemical properties of water and butanol, or on their interaction with an auxiliary agent or material. Some of the more investigated separation techniques are: adsorption (Qureshi et al. 2005), Nielsen and Prather 2009), pervaporation (Sirkar and Thongsukmal 2007, Van Hecke et al. 2018), gas stripping (Ezeji et al. 2005), Xue et al. 2012), perstraction (Abdehagh et al. 2014, Qureshi and Maddox 2005, Jimenez-Bonilla and Wang 2018).), vacuum flash separation (Mariano et al. 2011, Qureshi et al. 2014) or Liquid-liquid extraction (Roffler et al. 1986, Qureshi et al. 2005). Liquid extraction (LE) will be more deeply developed in next sections of this work.

Some hybrid processes combining at least two ISPR techniques have been also evaluated to profit each system's advantages in a synergic way. Some examples include the combination of both *in situ* recovery techniques (Gas Stripping and LE) and their applications for batch and fed-batch ABE fermentations (Lu and Li 2014).

Establishing a rank of ISPR techniques based on literature data is not an easy task, since experimental conditions and underlying assumptions vary between studies (Oudshoorn et al. 2010). Energy consumption is a key factor to evaluate and compare ISPR techniques. Nevertheless, in most cases the calculation details are not reported and the data focus only on the energy cost reduction of the downstream section. A more complete analysis of different ISPR techniques has been published by Outram et al. (2016). They compared the downstream energy economy against the energy demand of each ISPR technique. The authors concluded that the ISPR techniques increase profits over a conventional batch plant, even if they generally increase the overall energy requirement. Table 1.3 shows the estimation of energy requirements for different recovery techniques.

Table 1.3. Energy requirement of butanol recovery systems (N/A: not available data)

ISPR	MJ/kg BuOH[a]	MJ/kg BuOH[b]	MJ/kg BuOH[c]	MJ/kg ABE[d]
L-L extraction	8,8	N/A	9,9	180
Perstraction	N/A	14	N/A	99
Pervaporation	13,7	9	N/A	125
Gas Stripping	21,7	21	15,3	280
Adsorption	8,1	33	N/A	110

[a] (Qureshi et al. 2005). Energy requirement for butanol separation from the broth (acetone and ethanol not considered).

[b] (Groot et al. 1992). Energy requirement for the overall process, based in the 2-columns system (purification of acetone and ethanol not considered)

[c] (Salemne et al. 2016) Downstream section only. ISPR replaces the beer column, four distillation columns set up (separation of acetone, butanol and ethanol).

[d] (Outram et al. 2016) Total energy requirement is considered (integrated separation: removal and regeneration, downstream section and heating/cooling/pump duty). ISPR ABE rich-stream is added after the beer column, four distillation columns set up (separation of acetone, butanol and ethanol).

Despite the high number of research publications on the application of ISPR techniques and the advantages over the conventional straight forward scheme, almost all studies in the last decades were carried out at laboratory scale (< 10 L). There are only few examples of the application of ISPR technology in production plants. One of them is the recovery of biobased isobutanol by flash distillation (Gevo's integrated process, GIFT) introduced in an industrial plant of $68x10^6$ L/year capacity in Luverne (MN, USA).

More research is required to prove the scalability and long-term robustness of ISPR technologies (Van Hecke et al. 2014). Besides, rigorous integrated technical evaluations considering all process streams are also needed. In fact, the introduction of complex configurations in the ISPR design must improve its performance sufficiently to justify the investment and the increased risk associated with the introduction of novel technologies.

1.7 Extractive fermentation

In extractive fermentation, a water insoluble compound, with ideally high alcohol capacity, is added to the fermentation broth. Both phases are then easily separated.

The ABE fermentation can be associated to a Liquid-Liquid extraction operation in several schemes or setups *(in situ* or *in stream*, Figure 1.5). In both cases, the product is recovered "as quickly as it is formed" from the biocatalyst environment, and the fermentation may be carried out in batch, fed-batch and continuous operation. Comparisons between operation modes are applicable for extractive fermentations. For example, compared to a batch system, an extractive fed-batch configuration can transform much more concentrated substrates (from 60-80 g/l up to 300-500 g/l (Qureshi et al. 1992)) thus generating significantly less wastewater process. In the same way, a continuous extractive fermentation will allow diminishing lag times and increasing thus the global productivity, but this system will suffer from 'typical' problems of the ABE continuous fermentation (degeneration of strains, oscillations….) (Li et al. 2011) (Ennis and Maddox, 1989) (Gapes et al., 1996) In addition, the arrangement of the extractive fermentation process will depend on the microbial culture disposal: are the cells suspended inside the bioreactor, or immobilized? Are they recycled back to the reactor after an ultrafiltration step or passing through the separation unit? All these factors must to be considered in order to compare the different process arrangements in coupled fermentation-extraction systems.

The simpler configuration corresponds to an *in situ* extractive fermentation setup. In this case, the solvent is added to the bioreactor, which becomes a two-phase partitioning system. No additional extraction vessel is required; the fermenter acts as a single-stage extractor. On the other hand, fermentation conditions (temperature, pH) must be compatible with those of the Liquid-Liquid extraction. The microorganisms are in direct contact with the extracting solvent: therefore, the biocompatibility of the solvent must be evaluated and ensured.

Different multi-stage configurations were also reported in literature. Bankar et al. (2012) proposed to split the continuous ABE solvent production into two different reactors with an intermediary liquid-liquid extraction step allowing the recovery of the inhibitory product formed in the 1st stage. The authors used a mixture of oleyl alcohol and decanol as the extractant and attained an overall productivity of 2.5 g/ (L h) for a previously optimized dilution rate of 2.5 h^{-1}. This setup does not correspond rigorously to an ISPR system: the kinetics in each reactor is not enhanced by the product removal, since the latter doesn't occur concomitantly to the reaction, but in a separated stage.

Another “two-stage” process was proposed by Ramey and Shang-Tian, (2004). In this case, both subsequent phases of the ABE fermentation – acidogenic and solventogenic- take place in different bioreactors, allowing for higher flexibility (the main operating parameters accounting for specific kinetics of each phase). The use of an acid-former strain is reported in the first reactor leading to high yields in butyric acids, and the second reactor is fed with the acids formed in the first step along with supplementary glucose to provide energy enough to convert the acids into solvents. Liquid-liquid extraction concerns only the solventogenic phase, once the toxic products (butanol, acetone, and ethanol) start to be formed, thus preserving the extraction of the main intermediary product

More recent configurations of liquid-liquid extraction coupled ABE fermentation involves immobilization of the culture. In Wang et al. (2016), extraction occurs in a two-phase vessel placed *in stream* to a packed bed biofilm reactor, and butanol separation is enhanced by gas stripping directly through the organic phase. The authors announce a butanol productivity of 11 g/ (L h) in a continuous mode and a recovery up to 64% of the butanol produced with this configuration.

1.7.1 Solvent choice: general criteria and state of art

One of the most important issues in extractive fermentation process development is the search for high performance solvents. The extraction solvent should present adequate features for the recovery of fermentation products in order to minimize solvent consumption and product recovery costs. Nevertheless, one of the most severe constraints in the selection of a solvent might be the biocompatibility with the microbial cultures.

Some general criteria for a solvent in the case of an *in situ* product recovery system are listed here:

i. Good extraction performance. This is mainly determined by two parameters:

- The distribution or partition coefficient for the product, which is defined as the ratio of the product concentrations between solvent and aqueous phases. The greater the partition coefficient is, the lower the ratio of solvent to water required.
- The selectivity, defined as the ratio between product and water partition coefficient. A low selectivity implies water losses into the solvent phase, which may make difficult the final separation (the product recovery from the solvent phase).

ii. Biocompatibility, or low toxicity/inhibition towards the microorganism

iii. Effective product recovery from the solvent (easy regeneration). If the extracted products are recovered by distillation, the solvent should be less volatile

than the products so that large amounts of solvent will not have to be vaporized in the distillation column. The extraction solvent should not, however, be so nonvolatile that expensive high-pressure steam is required in the reboiler of the solvent regeneration column.

iv. Rapid phase separation, or density difference between the solvent and aqueous phases

v. Low solvent solubility in aqueous phase, to minimize solvent losses as well as waste treatment.

vi. No bioavailability: the solvent cannot be uptaken as a nutrient source by the microorganisms

vii. Low formation of emulsions and foams, which could impede the operation in a partitioning reactor and the further separation of phases.

viii. Viscosity of the solvent is important, since it influences the efficiency of extraction through its effect on mass transfer coefficients. A high viscosity increases also the requirement for pumping and mixing.

ix. The interfacial tension is an important parameter to be considered. If the interfacial tension is low, stable emulsions which difficult the phase separation risk to be formed; when the interfacial tension is too high, the energy requirement to maintain enough contact surface tends to be increased.

x. Good handling properties and low toxicity (workers, environment)

xi. Low cost and availability

The ideal solvent should possess all the previous characteristics, but in practice it is quite difficult to find a solvent fulfilling all these strict requirements. For example, higher distribution coefficients are usually accompanied by toxicity effects on microbial cultures while biocompatible solvents are in general high molecular weight and viscous compounds. Therefore, the task of solvent selection must result in a good compromise between all the criteria cited above. Additional specific criteria for ABE fermentation concern intermediates and co-products interaction. Indeed, an ideal extracting solvent should be able to co-extract the acetone and the ethanol, which are the more important by-products and could reach inhibitory levels in the fermentation broth longer operations. Furthermore, extraction of butyric acid – main intermediate produced during the acidogenic phase - should be a priori minimized: otherwise this intermediate would not be able to its further conversion into butanol in the solventogenic phase.

1.8 Aims and scope

The global objective of the research described in this work is formulated here:

To evaluate and quantify the performance of the ABE fermentation process coupled to an *in situ* solvent extraction phase. The specific aims deal with the comparison of a certain number of extraction solvents by means of their separation factor and biocompatibility issues.

Attention is focused on the assessment of the influence of the solvent, not only on the physical effects (according to thermodynamics) but also on the metabolism and microbial population dynamics evolution during batch and fed-batch extractive fermentations.

Butanol production within ABE extractive fermentation using two selected solvents of different properties in terms of separation capacity and biocompatibility will be techno and economically evaluated. The integration of this process within a LCB biorefinery using a 2G type substrate will also be considered.

1.9 Outline

This introduction outlines the importance of the industrial transition towards a biobased system and, in this context, the key role of butanol as an advanced biofuel and a building block for chemistry. Conventional ABE fermentation is described (microorganism, metabolic pathways, process limitations), and general research guidelines and strategies to improve old process performance are presented.

The first part of this Thesis (Chapter 2 and 3) focuses on the solvent selection, aiming at the identification of any solvent-dependent phenomena that might influence the process scale-up. Chapter 2 presents a global methodology for solvent screening and depicts useful trends for several chemical families studied, referring to physical (separation capacity) and physiological (biocompatibility, bioavailability) features. The behavior of Clostridia with two selected solvents of different nature is deeply evaluated in batch extractive fermentation in Chapter 3.

The aim of the second part of this work (Chapter 4 and 5) is to enhance comprehension of the process, by further analyzing the extractive ABE fermentation through the microbial population dynamics inside the bioreactor. In Chapter 4, the application of flow cytometry technique allows to account for the cell heterogeneity response in control and two different solvent-based extractive fermentation. Experimental information is used to develop a segregated ABE fermentation model, based upon a new cell cycle proposal. This is the object of Chapter 5.

In Chapter 6, a techno-economic evaluation of the global integrated process (fermentation + extraction, regeneration, downstream) is performed, based on the nature of the solvent and the operation mode applied (batch or fed-batch).

Experimental data are used as the input of simulations allowing to account for physiological response of the microorganism. The contribution of the different cost factors (raw material, OPEX, CAPEX) in different scenarios evaluated is discussed on the basis of the solvent properties.

Finally, the biorefinery concept is evaluated through the validation of a cellulose hydrolyzate from *organosolv* as a substrate for ABE extractive fermentation. In Chapter 7, results obtained with hydrolysate and synthetic medium are compared, and some strategies to enhance the fermentability of 2G-substrates in ABE fermentation are applied and discussed

2. Solvent selection

Solvent screening for *in situ* liquid extraction of products from Acetone-Butanol-Ethanol fermentation was carried out, taking into account biological parameters (biocompatibility, bioavailability and product yield) and extraction performance (partition coefficient and selectivity) determined in real fermentation broth. On the basis of different solvent characteristics obtained from literature, sixteen compounds from different chemical families were selected and experimentally evaluated for their extraction capabilities in ABE fermentation broth system. From these compounds, nine potential solvents were also tested for their biocompatibility towards *Clostridium acetobutylicum*. Moreover, bioavailability and differences in substrate consumption and total n-butanol production with respect to solvent-free fermentations were quantified for each biocompatible solvent. Product yield was enhanced in the presence of organic solvents having higher affinity for butanol and butyric acid. Applying this methodology, it was found that the Guerbet alcohol 2-butyl-1-octanol presented the best extracting characteristics (the highest partition coefficient (6.76) and the third highest selectivity (644)), the highest butanol yield (27.4%) and maintained biocompatibility with *Clostridium acetobutylicum* in batch screening experiences.

Part of this chapter has been redrafted after: Gonzalez-Peñas H., Lu-Chau T.A., Moreira M.T., Lema J.M. 2014. Solvent screening methodology for *in situ* ABE extractive fermentation. Applied Microbiology and Biotechnology 98(13):5915-5924

OUTLINE

2.1 Introduction

The solvent choice is one of the most important steps of the development of *in situ* extractive fermentations. Several works have focused on the research of suitable solvents for butanol extractive fermentations from the early eighties (Dadgar and Foutch 1985, Groot et al. 1990, Kim and Iannotti 1999). Oleyl alcohol has been reported by several authors to be one of the most performing butanol extracting agents in ABE fermentation (Roffler et al. 1988, Shukla et al. 1988, Dadgar and Foutch, 1985, Groot et al. 1990). It is a commercially available mixture of C-16 to C-18 unsaturated primary alcohols, with C-18 predominating. It is liquid at room temperature, and the major reported advantages are its non-toxicity towards Clostridium microorganisms and its high distribution coefficient for butanol (about 3-4 g/g). However, some authors reported some drawbacks in the use of oleyl alcohol in the ABE fermentation (Kraemer and Marquardt 2011): a) low partition coefficient for acetone (~ 0.3 g/g) that could enhance the inhibition phenomena in an industrial-scale process by the build-up of this co-product, b) relatively high viscosity (17 cP at 37°C) which might reduce mass transfer in the bioreactor, and c) high boiling point range between 382 and 349°C: high pressure steam would be required if the solvent is regenerated by distillation.

Other organic solvents were tested in ABE extractive fermentation. Polypropylenglycol was selected by Barton and Daugulis (1992), according to its butanol partition coefficient and biocompatibility, but resulted in decrease in yield (-21%) and productivity (-23%) in fed-batch fermentation. Dibutylphtalate (Wayman and Parekh 1987) was also tested but phase separation turned out to be difficult due to close density values of both phases. For an economically viable process it is essential to easily recover the extracted products, but solvent regeneration has not been discussed so much in the literature. To circumvent the regeneration, some authors investigated the use of extractants for direct use as biofuel. It is the case of the crude palm oil (Ishizaki et al. 1999), or the biodiesel (Li et al. 2010), with reported special emphasis in the enhancement of fuel properties (cetane index, cloud point...).

Appart from classical organic solvents, alternative systems have also been tested in ABE fermentation. Ionic liquids are interesting “designer solvents”, and some authors studied the 1-butanol extracting capacity of different imidazolium (Ha et al. 2010) or phosphonium and ammonium (Cascon and Chung 2011) based ILs. A review of the applications and perspectives of Ionic Liquids in industrial biotechnology reports that toxicity towards microorganisms constitiutes their main drawback and their flexible nature the most promising characteristic. (Quijano and Couvert 2010).

More recently, the recovery of ABE metabolites by supercritical CO_2 was succesfully tested at laboratory (Qureshi and Eller, 2017). Final fermenation broth is reused (allowing process water recycle) assuring that nutrients are not removed during the fermentation. Other authors reported the use of non-ionic surfactants to relieve microorganisms from butanol toxicity and consequent enhancement of butanol titer of 200% (Dhamole et al. 2012). Further optimization of process operating conditions (pH, surfactant concentration, immobilization of biomass) was achieved in Gedam et al.(2018)

Even if the search of an ideal solvent for ABE extractive fermentation has been the object of numerous reports, an integral methodology that considers different process parameters such as partitioning coefficient, selectivity value, effect of real broth composition, biocompatibility towards the specific microorganism, solvent bioavailability and product yield has not been described. Moreover, some inconsistency and relatively high variability is observed when comparing results from different sources (Offeman et al .2005).

The first general systematic methodology for selecting a suitable solvent for extractive fermentation was proposed by Kollerup and Daugulis (1985, 1986). The authors elaborated an Extractant Screening Program able to read a database of more than 1500 organic solvents, and to estimate their extraction capacities by means of UNIFAC coefficients. More sophisticated mathematical tools based on Computer Aided Molecular Design were also used in the last decade (Cheng and Wang 2010, Wang and Achenie 2002) to improve the search methods. Among the general criteria, the capacity of a solvent to extract the product to be recovered is considered the most important factor. It is often evaluated by the partition (or distribution) coefficient. The product selectivity (related to water) is less frequently evaluated, but it is also a key parameter to be included in such screening studies, since the coextraction of water with product could strongly impact the cost of the process development.

In addition to high product partitioning, a good solvent must have no toxicity towards microorganisms. Solvent toxicity is correlated with their hydrophobic character (less water affinity usually implies more biocompatibility), which is commonly expressed as the logP value (or $\log K_{\text{octanol-water)}}$. However, depending on the target molecule to be recovered, a high hydrophobic behavior could be inversely proportional to its extraction capacity. This is the case of more hydrophilic solutes, like ethanol or, in a less extent, butanol.

Therefore, the solvent selection for an extractive fermentation remains a complex task, and necessarily leads to a compromise between extraction capacity and biocompatibility. In this work, a solvent screening methodology is proposed to identify high performance solvents for liquid-liquid extraction coupled to the ABE

fermentation. Potential solvents belonging to different chemical families (alcohols, esters, oils and ionic liquids) were first identified in a pre-screening step based on data obtained from literature. Then, several selected were tested experimentally in order to determine their (a) partition coefficients and selectivity and (b) their biocompatibility with *Clostridium acetobutylicum* and bioavailability in a series of experiments based on microfermentations. In order to mimic a real system, a culture broth obtained from a 2L bioreactor fermentation with *C. acetobutylicum* was used as aqueous phase. Glucose consumption and total butanol production (in aqueous and organic phases) were compared to the results obtained in control fermentation (no solvent).

2.2 Materials and methods

2.2.1 Extractants

The selected solvents from each chemical group were five alcohols: 2-ethyl-1-hexanol (2E1H), 1-dodecanol, 2-butyl-1-octanol (2B1O), 2-hexyl-1-decanol (2H1D) and oleyl alcohol (oleyl alc), five esters: diisobutyl adipate (DiB adipate), dibutyl phtalate (DiB phtalate), tributyl citrate (triB citrate), dibutyl sebacate (DiB sebacate) and bis (2-ethylhexyl) adipate (B(2E1H) adipate), four oils: silicon, sunflower, pomace and castor oils, and two water-immiscible ionic liquids: methyltrioctylammonium (Aliquat) chloride and trihexyl (tetradecyl) phosphonium chloride (phosphonium). Some of their important properties of the selected candidates are included in (Gonzalez-Peñas et al. 2014). Our selection was based on reported extraction performance, estimated *a priori* biocompatibility (by logP value) and some other important criteria such as water solubility, density, availability and price. The ionic liquids were chosen by their hydrophobic character, and their availability at the laboratory

2.2.2 Microorganism and culture media

Clostridium acetobutylicum ATCC824 was used as the solvent producing microorganism. All experiments were started with a frozen spore suspension in saline solution. *C. acetobutylicum* spores (150 µL) were heated-shocked for 1 min at 100ºC in order to induce spore germination and then they were used to inoculate 10 ml of potato/glucose preculture medium, which was incubated anaerobically for 72h at 36ºC. The potato/glucose pre-culture medium contained per L: 250 g potato, 2 g $(NH_4)_2SO_4$ (Panreac), 2 g CaCO3 (Panreac) and 10 g glucose.

The pre-culture medium was transferred to 100 ml of culture medium disposed in a 250-ml sealed flask, which was previously purged with N_2. The culture medium composition per L was as follows: 6.6 mg $FeSO_4x7H_2O$ (Panreac), 0.56 g $MgSO_4x1H_2O$ (Fluka), 1 g KH_2PO_4 (Panreac), 0.6 g K_2HPO_4 (Panreac), 2.9 g

CH_3COONH_4 (Panreac), 0.1 g p-aminobenzoic acid (Sigma Aldrich), 2.5 g yeast extract (Sigma Aldrich) and 60 g glucose (Merck). The flask was incubated at 36°C in a shaker with gentle agitation.

In the case of microfermentations for biocompatibility evaluation, 12-ml tubes filled with 4 ml culture medium were inoculated with cells growing at a high rate. The initial glucose concentration was fixed at 90 g/l, to allow higher substrate consumption in effective extractive fermentations. Experiences were carried out at 36 °C.

All of the fermentations were carried out without pH control: the pH value was adjusted to 6 at the beginning of the fermentation, and then it varied freely through the fermentation according to the acid generation and subsequent consumption. All of the mediums and material were previously autoclaved.

2.2.3 Bioreactor

In order to generate enough volume of representative fermentation broth, a batch fermentation was carried out in a 2-L stirred tank reactor BIOSTAT B (Sartorius Stedim Biotech SA), filled with 1.2L of culture medium. The liquid was purged with pure N_2 for 1 hour previously to inoculation with 100 ml of cells growing at µmax (maximal growth rate). The fermentation was carried out without pH control, and at low stirring rate (80 rpm). The temperature was fixed at 36ºC. The whole system was autoclaved at 121 °C during 20 minutes before inoculation. Salts and glucose of the initial fermentation medium were autoclaved separately in order to avoid Maillard reactions during this operation.

The broth was recovered at the end of the fermentation by centrifugation at 5000 rpm during 10 min, and the supernatant was stored at -20ºC. Before the preparation of the extraction runs, the broth was filtered through 0.22 µm membrane to avoid contaminations during the extraction tests.

2.2.4 Extraction performance parameters evaluation

The extraction performance of each solvent was evaluated considering two parameters: the partition coefficient (K) and the selectivity (SEL).

The partition coefficient was calculated according to equation (2.1).

$$K_{BuOH} = \frac{[BuOH]_{org}}{[BuOH]_{aq}} \qquad (2.1)$$

where $[BuoH]_{org}$ and $[BuoH]_{aq}$ are the equilibrium butanol concentrations in the organic and the aqueous phase, respectively

The selectivity (or separation factor) for a compound is expressed as the division of the distribution coefficient of that compound by the water distribution coefficient:

$$Sel = \frac{K_{BuOH}}{K_{water}} \tag{2.2}$$

Shaken tubes method was used for the partitioning experiments. The extractions were performed in 1.43 x 12 cm conical graduated polyethylene tubes, with an aqueous to organic phase volume ratio of 2:1, and total liquid volume of 7.5 ml. It can be seen from most of ternary diagrams that equilibrium composition between phases are not expected to vary substantially with amount of solvent added when initial aqueous mixture are highly diluted (maximum 2%wt of solvent in ABE fermentation case).The tubes with the liquid mixture are placed in a shaker previously heated up to 36ºC, and then are shaken vigorously in horizontal position to completely emulsify both organic and aqueous phases for 2 h. The tubes can settle vertically for 24 h.

The necessary time to reach equilibrium conditions depends on mass transfer rates between phases, which are function of viscosity of both phases, the specific interfacial area between phases (S/V) and fluid motion. A way to increase this specific surface may be the creation of small droplets (with high surface to volume ratios) by emulsification of the phases. This can be achieved by repeated vigorous agitations.

To clear both phases and to break wall droplets or emulsions, the tubes are centrifuged during 20 min, at 5000 rpm. For the more viscous solvents, this harvesting step can last longer to complete separate two clear phases.

An aliquot of the organic phase (the upper phase in most cases) is removed and stored for further analyses. After discarding the boundary layer between both phases, an aliquot of the aqueous phase is separated in another tube with the help of a long pinhead syringe. Sampling with pipette of the phases must however be done carefully in order to not mix phases at this stage.

The organic phase concentration for butanol and the other metabolites was estimated by solute mass balance, as follows:

$$[BuOH]_0 \cdot V_0 = [BuOH]_{aq} \cdot V_{aq} + [BuOH]_{org} \cdot V_{org} \tag{2.3}$$

with:

$$\frac{[BuOH]_0}{[BuOH]_{aq}} \cdot V_0 = V_{aq} + K_{BuOH} \cdot V_{org} \quad (2.4)$$

The performance of the extraction is evaluated by the analytical quantification of final aqueous products (butanol, acetone, ethanol, acids). It was assumed that the solubility of the solvent is negligible. Taken this into account, the water concentration in aqueous phase was estimated using (5):

$$[H_2O]_{aq} = 1 - \sum_i [C_i]_{aq} \quad (2.5)$$

where i: 1 to number of compounds in the aqueous broth (butanol, acetone, ethanol, acids. glucose…). Standard deviations (SD) of both parameters were estimated from results of triplicate extraction runs.

The water content of the organic phase was evaluated by Karl-Fischer titration (Metrohm 795 KF). This is necessary to calculate the selectivity.

2.2.5 Biocompatibility evaluation

Solvent toxicity towards *C. acetobutylicum* was evaluated by comparing glucose uptake and total butanol production between control and solvent-exposed microfermentations carried out in duplicate. Tests were conducted in 12 ml PE tubes, with an aqueous to organic phase ratio equal to 1:1, and a total volume of 8 ml.

The tubes, with 4 ml of growth broth and the same volume of extractant, were placed in an anaerobic jar for 24 h previously to inoculation. All the tubes were inoculated at 10% (v/v) with the same fermentation culture. The microfermentation tubes were covered with cotton and put again vertically in the anaerobic jar without agitation and were incubated at 36°C during 72 h. Then, the tubes were centrifuged during 20 min at 5000 rpm and both phases were separated for further analysis.

2.2.6 Bioavailability determination

Potential solvent bioavailability to *C. acetobutylicum* was tested in independent experiments conducted at the same conditions as those previously described for evaluating biocompatibility. However, in these experiments, culture medium was formulated without the addition of glucose. For considering that a solvent could be used as a carbon source by the bacterium, the net change in cell density at the end of the fermentation was compared to the change observed in a negative control formulated without the addition of solvents (Janikowski, Velicogna, Punt, and

Daugulis, 2002). Solvents that presented growth significantly higher than that achieved by the negative control were considered bioavailables (+), while those that were below that value were non-bioavailables (-).

2.2.7 Analytical procedures

Aqueous and organic phase compositions were determined by gas chromatography (GC) (HP6890 GC) with N2 as the carrier gas and a flame ionization detector. The GC was equipped with an Agilent 122-7032E column. The oven was kept isothermally at 70°C for 3 min and thereafter, temperature was increased up to 200°C at a gradient of 50°C/min.

To estimate the water content in aqueous phase, it was assumed that the aqueous solubility of the solvent was negligible. In the case of organic samples, hexane was combined at a ratio of 1:1 vol, to the organic sample being analyzed. The objective is to ensure a single homogeneous sample at room temperature before GC analysis. Only butanol was quantified in the organic phase at the end of microfermentations for biocompatibility evaluation. The water content of the organic phase was evaluated with a Karl Fischer titration (Metrohm 795 KF). Quantification of glucose was performed using an enzymatic kit (Glucose-TR GoD-PoD, Ref.1001190, Spinreact, GI, Spain).

Biomass growth was estimated by measuring the optical density of an aqueous phase sample at 600 nm in a spectrophotometer. This sample was obtained after centrifugation of the culture broth at 5000 rpm for 10 minutes and subsequent wash of the biomass pellet with distilled water.

2.2.8 Statistical analysis

Statistical analysis was conducted using the software R (R Development Core Team, 2009) to verify the existence of significant differences and to carry out control-to-solvent and pair-wise multiple comparisons among all solvents ($p_{\_,s}$), solvents within a specific solvent group ($p_{\_,g}$) or mean values of solvent groups ($p_{\_,m}$). First, a one-way analysis of variance using parametric (ANOVA ($p_{A,\_}$), based on a linear model) and non-parametric (Kruskal-Wallis, ($p_{K,\_}$)) methods was carried out to determine if the values obtained using different solvents or group of solvents were significantly different. Then, if a difference between the mean values was confirmed, a post-hoc analysis was applied to determine the values for which that difference was significant, considering a level of significance of 0.05. Dunnett/Tukey ($p_{D,\_}$) non-parametric test (R-package *nparcomp*) was applied when the number of samples was different between groups and/or the assumption of homogeneity of variances was not met. In all other cases a Tukey-HSD ($p_{T,\_}$) test was carried out.

2.3 Results

2.3.1 Theoretical approach: general trends

An initial list of 63 potential solvents was developed according to bibliographic research on extractive ABE fermentation and equilibrium data. This database can be found as Supplementary Material in Gonzalez-Peñas et al. (2014). It regroups the different chemical families that present affinity for butanol in an aqueous system, and compile the results obtained with different solvents already tested in ABE or ethanol extractive fermentations. Qualitative or quantitative information including cost, handling properties, density, viscosity and other properties was also considered in the pre-screening. Even if these data are intrinsically heterogeneous, since the experimental procedure changes for each research work, and should not rigorously be compared quantitatively, some general trends are evidenced from the compiled data.

The selected indicator for biocompatibility assessment was the logP (log K_{ow}). When this value was not available in literature, it was estimated by using the Rekker's group contribution method (Rekker, 1977).

As a general rule, alcohols present the best extractability for butanol in terms of the highest distribution coefficient (K) which range from 3 to 8 (on a mass basis), when looking at those alcohol-based compounds presenting logP > 3. The distribution coefficients for butanol and ethanol decrease as the concentration of alcohol hydroxyl groups decreases, which means, when alkyl chain length or molecular weight of the solvent increases.

Selectivity presented higher variability, thus direct conclusions from literature data are more difficult to obtain. This higher variability can be due to intrinsic larger measure errors (selectivity is estimated from at least three measured concentrations, and water partition coefficient tend to be very low for the solvents considered, implying near to the detection limit analysis and division by quite small numbers).

Esters and ketones go second in terms of distribution coefficient for butanol. From the oils presented in the database, castor oil presents the highest distribution coefficient for butanol (3.6 g/g) while the rest of the oils present distribution coefficients lower than 1. Alkanes present the lowest distribution coefficient for butanol, acetone and ethanol, and do not appear suitable for the liquid-liquid extraction of the fermentation products. However, their use in solvent mixtures may be justified for improving other important criteria (e.g. viscosity).

After applying the prescreening, 16 chemicals were selected to be experimentally evaluated for their extraction capacity in ABE fermentation broth. These include five alcohols, five esters, four oils and two ionic liquids.

2.3.2 Distribution coefficients and selectivity values

The values for butanol selectivity over water and butanol distribution coefficients obtained experimentally are given in Table 2.1. The initial butanol, acetone and ethanol concentrations in the aqueous fermentation broth were 10.62, 5.43 and 1.52 g/L, respectively. The complete set of results concerning equilibrium partition coefficient for intermediates (acids) and co-products (acetone, ethanol) of the ABE fermentation, and their selectivity values are presented as Supplementary material in Gonzalez-Peñas et al. (2014).

Table 2.1. Extraction results at 36ºC and [BuOH] = 10.6 g L $^{-1}$, and comparison with literature data

	This work		Literature			
	KBuOH(SD)	Sel BuOH(SD)	K BuOH	Sel BuOH	T(°C)/[BuOH]	Reference
Alcohols						
2-butyl-1-octanol	6.76(0.25)	644.8(23.5)	N/A	N/A	N/A	---
Oleyl alcohol	4.57(0.21)	294.7(11.3)	3.2	N/A	34/1.2%wt	Evans et al 1988
			4	N/A	24/1.5%wt	Malinowski et al 1994
			3.3	N/A	30/1.5%wt	Ishizaki et al 1999
2-ethyl-1-hexanol	7.95(1.52)	311.1(61.6)	6.1	276	28/2%vol	Kim et al 1990
2-hexyl-1-decanol	3.41(0.27)	509.2(36.8)	N/A	N/A	N/A	---
1-dodecanol	5.06(0.32)	171.5(7.1)	5.1	N/A	34/1.2%wt	Evans et al 1988
			6	140	25/1.5%wt	Groot et al 1990
Esters						
Diisobutyl adipate	2.6(0.07)	834.1(24.4)	2.5	3	34/2%wt	Groot et al 1990
Dibutyl sebacate	1.89(0.02)	474.0(4.2)	1.8	N/A	37/1%vol	Barton et al 1992

	This work		Literature			
	KBuOH(SD)	Sel BuOH(SD)	K BuOH	Sel BuOH	T(°C)/[BuOH]	Reference
Dibutyl phtalate	1.91(0.12)	36.3(4.4)	1.8	N/A	37/1%vol	Barton et al 1992
			1.4	4	37/2%wt	Groot et al 1990
Bis (2E1H adipate)	1.83(0.05)	668.3(80.9)	1.6	1.6	37/1%vol	Barton et al 1992
Tributylcitrate	1.67(0.11)	73.7(11.9)	2.4	N/A	37/2%wt	Groot et al 1990
Oils						
Sunflower oil	0.44(0.07)	623.4(107.0)	N/A	N/A	N/A	---
Silicon oil	0.59(0.04)	3161.9(8.8)	N/A	N/A	N/A	---
Pomace oil	0.62(0.12)	577.6(124.7)	N/A	N/A	N/A	---
Castor oil	2.85(0.46)	285.7(60.3)	2.6	270	37/2%wt	Groot et al 1990
Ionic liquids						
Aliquat 336	8.86(0.91)	41.7(3.8)	N/A	N/A	N/A	---
Phosphonium	11.55(0.64)	83.0(2.1)	N/A	N/A	N/A	---

N/A not available

Distribution coefficients range from 0.4 to 12 and compare reasonably well with data available in literature. Highest distributions coefficients were found for the ionic liquids ($p_{T,m}<1x10^{-7}$) and the lowest values correspond to oils and esters ($p_{T,m}$ =0.245). In general, the chemical families tested can be ranked as follows: ionic liquids > alcohols > esters ~ oils.

Considering selectivity, silicon oil presented the highest value (Sel > 3000). This oil consists in a polymerized siloxane, whose structure contains silicon, oxygen and an alkane side chain, which confers strong hydrophobic properties. On the other side, ionic liquids, two high density esters (dibutyl phtalate and tributyl citrate) and one alcohol (dodecanol) presented the lowest separation factors (p_T>0.187 between them, and p_T<0.0052 compared with the other solvents). Few data for selectivity values is available in literature.

Some trends are revealed when selectivity is plotted against distribution coefficients for the oils, esters and alcohols datasets separately. Figure 2.1 displays

butanol extraction results obtained for alcohols, which are one of the better classes of solvent in terms of distribution coefficient. 2-ethyl-1-hexanol presents the highest partition coefficient. However, it cannot be considered as a biocompatible *a priori* solvent (log P <3). It is proposed at this step as a potential solvent to be used in mixtures.

Distribution coefficients of esters (Figure 2.1) fall within a relatively narrow range, and are significantly lower than those obtained for alcohols ($p_{K,m}<1x10^{-5}$). Dibutyl phtalate and tributyl citrate present the lowest value for selectivity ($p_{T,g}=0.753$ between them, $p_{T,g}<0.0025$, when compared with the other esters). In addition, the density of these extractants is higher than that of water. This phase inversion may difficult the operation at higher scale.

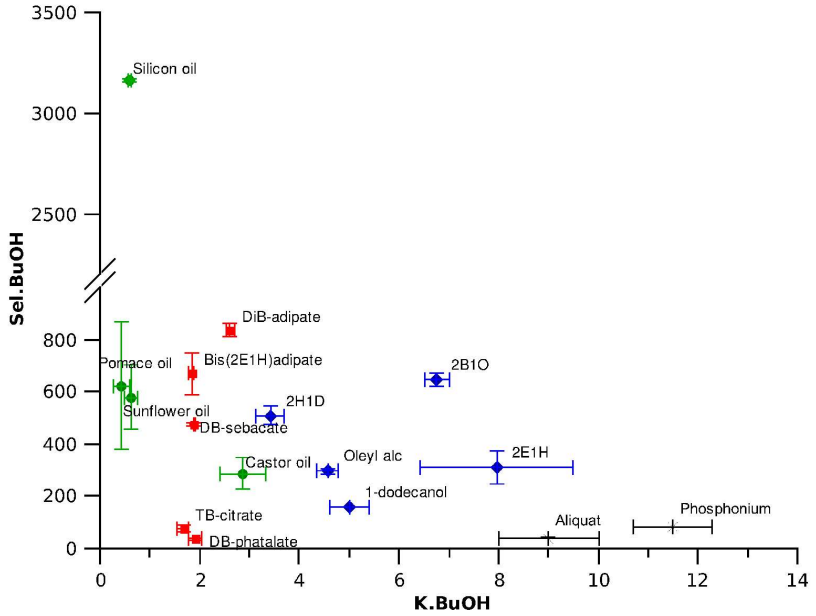


Figure 2.1. Distribution coefficients (K) and selectivity of butanol on A) five alcohols (blue diamonds), B) five esters (red squares), C) four oils (green circles) and D) two ionic liquids (grey cross).

Three vegetable oils and one silicon oil were tested. Castor oil presents the highest K_{BuOH} (2.6) *vs* the others oil tested ($p_{T,g}<2x10^{-5}$). However, its low selectivity and high viscosity (1500 cP) makes the use of this oil less attractive in commercial purposes. Viscosities of the other three oils are in the range 10-85 cP. Silicon oil presents the highest value for selectivity of all the solvents tested due to its strongly hydrophobic chemical structure (siloxane polymer). Pomace and sunflower oil form

a group with a K coefficient near to 0.5 and selectivity around 600 ($p_{T,g}$ for K and Sel were 0.798 and 0.916, respectively).

Results obtained with the ionic liquids are depicted in Figure 2.1. The highest distribution coefficients ($p_{D,m}<2x10^{-4}$) were obtained compared to the other solvents. K_{BuOH} values are approximately 3-4 times higher than that obtained for oleyl alcohol. Lowest selectivities were also obtained by ionic liquids. These values were similar to that obtained with esters ($p_{D,m}=0.178$), but significantly lower than those of alcohols and oils (pD,m<1x10-6). The two ionic liquids investigated present an extremely high viscosity (~1800 cP at 25 °C)

For the purpose of limiting the set of potential solvents, and based on extraction performance characteristics and other observed parameters during the extraction runs (viscosity, emulsion formation, etc), two alcohols (oleyl alcohol and 2-butyl-1-octanol), two esters (diisobutyl adipate and bis (2-ethyl-1-hexyl) adipate) and three oils (pomace, sunflower and silicon oils) were selected to be further screened for their biocompatibility with *C. acetobutylicum.* In addition, a mixture containing 20/80 (v/v) of 2-ethyl-1-hexanol and Pomace Oil respectively was also investigated. The objective was to determine whether this mixture could improve the overall distribution coefficient of the vegetable oil having poor affinity for butanol (K=0.4), keeping low aqueous concentration of the toxic solvent in order to not harm the biocatalyst.

2.3.3 Biocompatibility and bioavailability tests in microfermentations

Biocompatibility evaluation of nine solvents was experimentally determined in microfermentations. Glucose consumption is presented in Figure 2. It is observed that all the solvents, except 2-ethyl-1-hexanol (2E1H), are biocompatible. Standard deviations (SD) of glucose consumption for solvent-free control and for all microfermentations were lower than 3, except for dodecanol and diisobutyl adipate (12 and 30, respectively). Dodecanol has already been reported to be toxic towards *C. acetobutylicum* (Evans and Wang, 1988), and some inconsistency with the use of this solvent was also highlighted for ethanol extractive fermentation (Offeman and Stephenson, 2008)

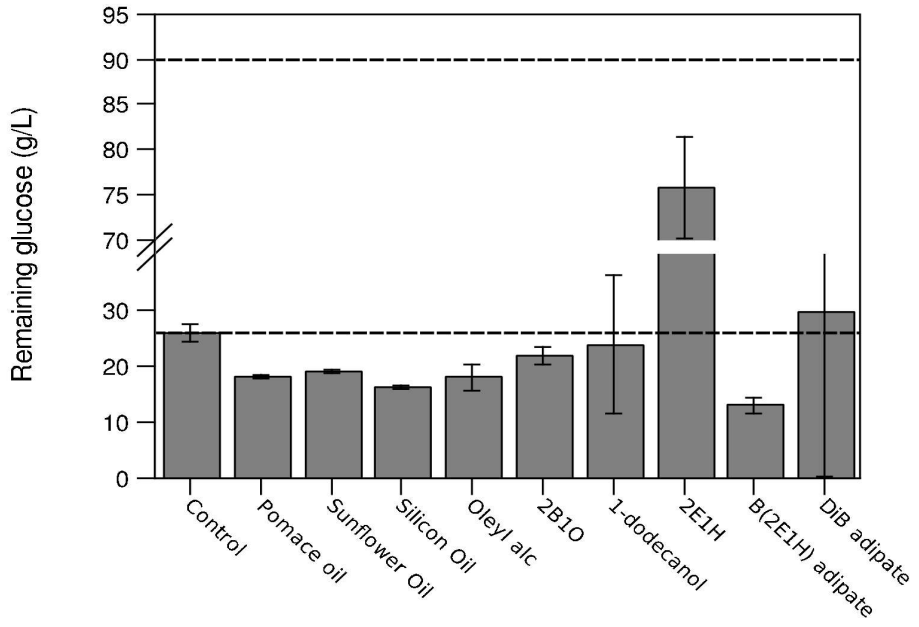


Figure 2.2. Remaining glucose in screening microfermentations

In this work, log P values of diisobutyl adipate and dodecanol (4.19 and 4.99, respectively) are into the intermediary region, near the biological threshold. Glucose consumption for these solvents was similar to that of the control fermentation (p_D>0.999, *control-to-solvent comparison*). Nevertheless, due to the high SD, these compounds will not be considered. The other solvent falling in the log P critical zone, 2-butyl-1-octanol, with a logP of 4.99, resulted to be biocompatible. In Figure 2.3, both parameters, logP and glucose consumption, are represented. Log P is inversely correlated with alcohol distribution coefficient, and consequently, it should be convenient to work as near as possible to the biological threshold of the microorganism.

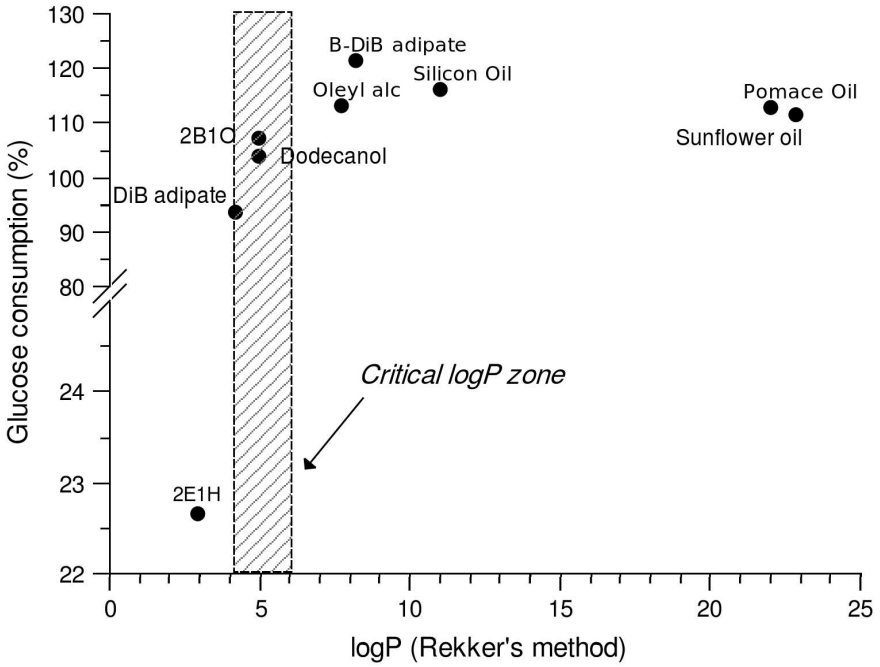


Figure 2.3. Glucose consumption over control fermentation vs estimated logP value of solvents investigated

At the end of the microfermentations, liquid-liquid equilibrium approach conditions are evaluated for all of the solvents tested. This can be observed in Figure 2.4, in which a comparison between experimental data (total butanol produced in both phases) and theoretical equilibrium partition for butanol (using K_{BuOH} estimated from the extraction runs) is presented. Some of the solvents (2E1H, vegetable oils...) strongly approached equilibrium conditions in spite of not advantageous geometry (high length/diameter) or lack of agitation.

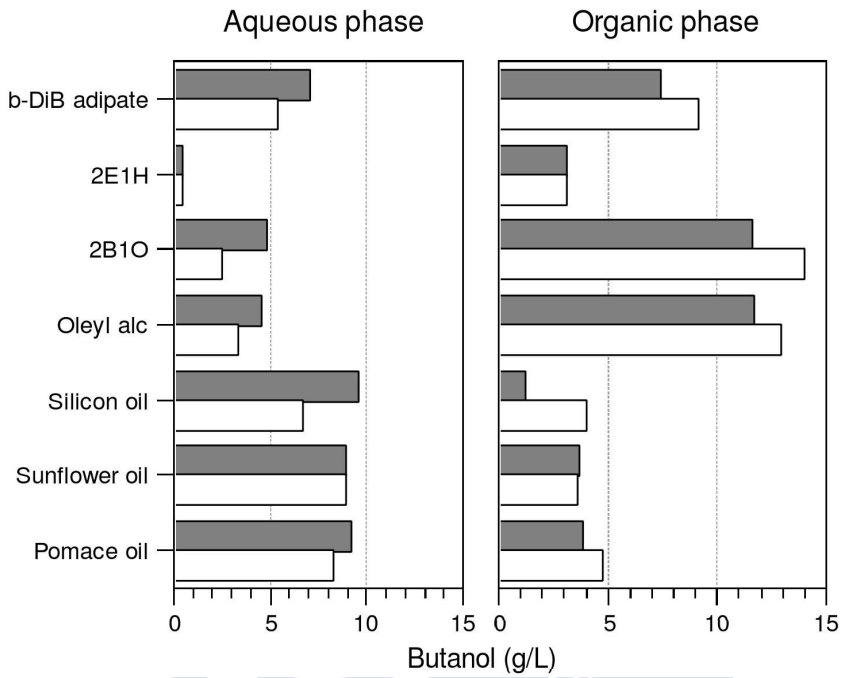


Figure 2.4. Partition of butanol into aqueous and organic phases (gray) comparison to equilibria data (white)

Figure 2.5 displays glucose consumption and butanol production over the control fermentation for the biocompatible solvents. Oleyl alcohol and 2-butyl-1-octanol present the highest final yields for butanol ($Y_{BuOH/Glu}$, in %w/w) (25.50 and 27.43, respectively, $p_{T,s} < 8x10^{-5}$), while silicon oil presents the lowest yield (16.45, $p_{T,s}<8x10^{-4}$). Adipate ester and vegetable oils resulted in similar yields (21.03 and 20.05-20.51, respectively, with $0.707<p_{T,s}<0.987$ between them).

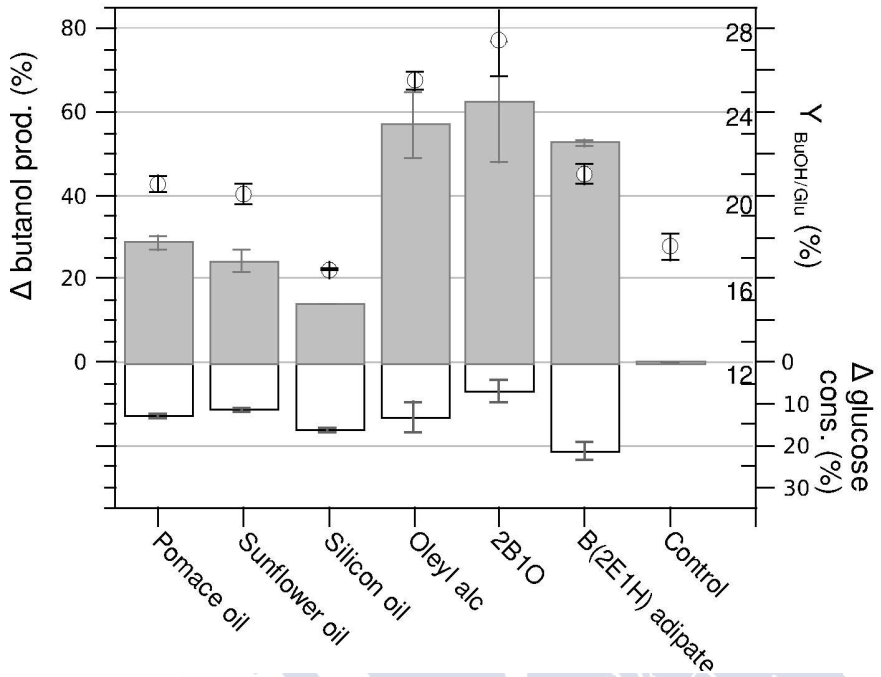


Figure 2.5. Increase of glucose consumption (white bars) and butanol production (gray bars) over control fermentation. Yield of butanol production with respect to consumed glucose is also shown (void circles)

The results obtained from microfermentations to determine the degree of biocompatibility are compiled in Table 2.2 Bioavailability of the solvents (or their potential to be consumed as a carbon and energy source by *Clostridium acetobutylicum*) was tested as a part of the solvent selection process, and the results are shown in Table 2.2. Only pomace oil showed cell growth significantly greater than the solvent-free reference (p_D = 0.0045).

Table 2.2. Butanol extraction performance, biocompatibility and bioavailability results

	BuOH extraction performance			
	K (S.D)	Sel. (S.D)	%Δgluc.(S.D)	Bioavailability
Pomace oil	0.63(0.12)	577(124.7)	12.8(0.6)	+
Sunflower oil	0.43(0.07)	623(107)	11.5(0.4)	-
Oleyl alcohol	4.57(0.21)	295(11.3)	13.2(3.4)	-
2-butyl-1-octanol	6.76(0.25)	644(23.6)	7.0(2.6)	-
Bis(2-ethyl-1-hexyl) adipate	1.83(0.05)	668(80.9)	21.3(2.2)	-
Silicon oil	0.60(0.04)	3161(8.85)	16.2(0.4)	-

2.4 Discussion

It has been stated that solvents with a logP value lower than 4 are, in general, toxic to *C. acetobutylicum* (Bruce and Daugulis, 1991). A solvent with a log P higher than 6 would preserve bacterial bioactivity, while for values between 4 and 6 it is difficult to conclude. As selection criterium, in this work it was decided to select solvents presenting log P values equal or higher than 4, which were defined as *a priory* biocompatible solvent. For potential biocompatible solvents presenting acceptable affinity for ABE fermentation products (in terms of K and selectivity values), other important characteristics were examined: density difference with water, solubility, viscosity, etc. Solvents presenting clear unacceptable properties were discarded, and the best ranked solvents of each chemical family were selected for further experimental screening.

It was reported that selectivity for ethanol is affected by the level of branching of the molecule and/or the OH position (Offeman and Stephenson, 2008). For the case of butanol, selectivity substantially increased if the solvent alcohol is beta branched or the hydroxyl group is placed in the middle of the molecule. The enhancement of the selectivity in more highly branched secondary and tertiary alcohols is explained by an extended hydrogen bond structure in the molecule and as a result of the changes in basicity caused by steric effects of the branched chains (Kim and Iannotti, 1999)

Ketones mix well with water and mutual solubility is more important than for the other chemical families. Thus, it is expected that important amounts of water

would be absorbed into the ketone (organic) phase during the liquid-liquid extraction of aqueous ABE broth. Their higher solubility in water implies also less biocompatibility *a priori.*

The vegetable oils are presented in a different chemical family, even when these triglycerides extracted from plants can be considered fatty esters. There are few data related to the extraction capacity of these compounds towards alcohol, but some authors reported the non-toxicity of vegetable oils towards *Clostridium* (Groot, Soedjak, and van der Lans, 1990). Other criteria such as viscosity – and then energy requirement for stirring and pumping – must also be taken into account when dealing with oils as potential extractants.

Other solvents such as halogenated hydrocarbons were reported to enhance ethanol and acetone extraction, but not so much butanol (Dadgar and Foutch, 1985). Many of these compounds have moderate to high toxicity, which makes them not suitable for any extractive fermentation application.

In this work, sixteen chemicals were experimentally evaluated for their potential use in ABE extractive fermentation. Two thermodynamic parameters (distribution coefficient and selectivity of the ABE products on the extractant solvents) and three biological parameters (glucose consumption, total butanol production and solvent bioavailability) were determined.

2.4.1 Distribution coefficients and selectivity

The composition of ABE fermentation broth may influence the solute mass transfer rate and the equilibrium data. It has been reported that the production of high titers of a biosurfactant (bacteriocin-like autolysin) at the end of the exponential growth phase (Webster et al., 1981). These compounds could reduce the extraction efficacy up to 10-15% (Pursell et al., 2004). Taking into account the effect of the culture broth composition, the extraction performance study was carried out using a representative culture broth obtained from an ABE fermentation with *C. acetobutylicum*.

Oleyl alcohol is the most studied solvent for *in situ* ABE extractive fermentations. Values of K_{BuoH} were reported as 3.2 (Evans and Wang, 1988), 4 (Malinowski and Daugulis, 1994) and 3.3 (Ishizaki et al., 1999). Variation between these values and that reported in the present study (4.5) may be due to differences in solvent purity, operating conditions, aqueous broth composition or analytical methods employed.

Influence of the branching degree is observed when comparing the selectivity of the isomers 1-dodecanol and 2-butyl-1-octanol (171 and 645, respectively). Increase of selectivity by location of the hydroxyl group in the middle of the molecule has already been reported for ethanol liquid-liquid extraction (Offeman

and Stephenson, 2008). Other important advantage of branched high molecular weight alcohols is that they are liquid at room temperature, whereas the unbranched n-alcohols are solid. Considering the adipate and sebacate based esters, some correlation appears again between branching degree and selectivity. In fact, dibutyl sebacate, which presents the lowest selectivity of the three compounds, is the only one having no branching in both side chains.

With respect to oils, high butanol affinity in castor oil can be explained by its predominant fatty acid structure which is the ricinoleic acid. It has an additional 12-hydroxyl group, conferring to the molecule supplementary hydrogen bonding site. This gives also affinity for water, explaining its lower selectivity. The similar performances of pomace and sunflower oils are due to their common dominant fatty acid structure: linoleic acid (double unsaturated, higher proportion in sunflower oil) and oleic acid (single saturated, higher proportion in olive oil).

The two ionic liquids assayed in this study present an extremely high viscosity (~1800 cP at 25 °C) which could represent an important drawback to their use for an *in situ* extractive configuration. Other important characteristic was their ability to form a 'third phase' between the aqueous culture broth and the ionic liquid at the end of each extraction run. A similar behavior was observed by Oliveira et al. (2012) during the extraction of organic acids with phosphonium-based ionic liquids from an aqueous solution. The formation of stable association of solvent molecules (clusters) at the solvent/aqueous interface might difficult the operation and entrain some quantitative solvent losses.

From an engineering perspective, an optimal balance between K_{BuOH} and selectivity will depend on process configuration (extraction and regeneration mode) and final product specifications. Operational cost generated by energy demand may be important in ABE extractive fermentation system given the latent heat of vaporization of water (>2400 kJ/kg) relative to that of butanol (~700 kJ/kg). Selectivity towards water is therefore a key parameter considering the highly diluted fermentation broth. On the other hand, solvent to feed ratio is an essential factor that affects capital expenditure and size of the equipment.

2.4.2 Biocompatibility and bioavailability

Whether *in situ* or *in stream* configuration system, the contact between the solvent and the microorganism is unavoidable in an extractive fermentation. Therefore, biocompatibility towards the specific biocatalyst must be guaranteed in such a system. An extended explanation about the mechanism of different types of toxicity (dissolved or biphasic toxicity), their effects on cell's membrane, and potential ways to predict the biocompatibility *a priori* can be found in previous works (Vermue and Tramper, 1995, Salter and Kell, 1995).

Another important factor to be considered is the agitation rate. Increased agitation may shift the critical threshold to the right, increasing the presence of toxic solvent, as result of better transfer into the aqueous phase (Bruce and Daugulis, 1991). A compromise must be achieved between mass transfer and bioactivity preservation.

The extent to which a solvent is biocompatible or toxic towards a microorganism can be determined by measuring the percentage of surviving cells, cell density, substrate utilization or product formation. In this work, glucose uptake and butanol production were the criteria chosen for screening solvent biocompatibility. Other parameters, like cell survival or cell viability are important criteria in mid-long-term fermentations and should be considered in further steps of solvent selection. Indeed, if metabolic activity is maintained, but low cell survival is observed, this can be explained by an initial harmless solvent action, when intracellular solvent concentration is not high enough yet. With time, the accumulation of solvent molecules could accelerate enzyme denaturation and eventually lead cell to death.

In this work, increased glucose consumption for biocompatible solvents with respect to control fermentations, did not lead systematically to the same overproduction of butanol. This fact reveals some influence of the solvent used in liquid extraction on ABE metabolism. Besides, $Y_{BuOH/Glu}$ reveals correlation with butanol and butyric acid distribution coefficients between phases. Some hypotheses that could be formulated in order to explain these results are the following:

a) The organic phase may act as a source of butyric acid during the solventogenesis (previously extracted during acidogenesis), modifying product yield structure. It has been reported that butyric acid addition during solventogenic phase enhances butanol production (Tashiro et al. 2004).

b) Acidic conditions favor solventogenic behavior and butanol metabolic pathway is stimulated at low pH (Li et al. 2011). Butyric acid from organic phase would have created NADH pressure by lowering the pH after the metabolic switch. This redox imbalance would enhance product yield, since oxidation of NADH is coupled to butanol and ethanol production, but not to acetone's.

c) Lower aqueous concentration of butyric acid during acidogenic step could have preserved to a certain extent the solventogenic role of the cells.

d) Increasing the efficacy of butanol removal from the aqueous phase could have improved specifically the butanol metabolic pathway by kinetics or thermodynamic means (or both).

Detailed profiles of biphasic fermentations with different types of solvents appear necessary to understand the relation between solvent nature and ABE metabolic pathways, to evaluate these hypotheses.

Finally, another important aspect to be considered for selecting a solvent is its bioavailability towards the fermenting microorganism. If the extracting solvent is readily used as a carbon source, fresh solvent addition might be necessary for compensating its consumption by the microorganism. This could represent an important economic drawback, whose significance will depend on the cost of the solvent. In this study, the criterium used for deciding if a solvent is bioavailable was that reported by Janikowski et al. (2002). According to this criterium, pomace oil would be a bioavailable solvent because cell growth higher than that of the negative control (without other carbon source) was achieved. However, even though statistically significant, the difference with the control was quite small. In another study, Anvari and Khayati (2009) considered as bioavailable those solvents that yielded cell growth of 15% or more of the growth observed in the negative control. Applying this latter criterium, pomace oil would be a non-bioavailable solvent. Moreover, in bioavailability assays, conditions are pessimistic over extractive fermentation process since the solvent is not competing with glucose to be consumed as substrate by cells. Based on all this information, we considered that pomace oil should not be excluded from subsequent studies, and that further experimentation is needed to obtain detailed information about its potential use as a substrate in culture media.

2.5 Conclusions

Sixteen chemicals were evaluated for their potential for use in ABE extractive fermentation. These compounds had been previously chosen based on theoretical considerations (literature data, properties…) and estimated biocompatibility *a priori* (logP value).

In a first step, an experimental database with partitioning performance of ABE metabolites in 'real' conditions (temperature, medium composition) was generated. Two parameters were evaluated for butanol and co-products (acetone, ethanol): distribution coefficient and selectivity value. Highest distribution coefficients were found for ionic liquids and the lowest values for oils. In general, the chemical families tested can be ranked as follows in terms of distribution coefficient: IL > alcohols > esters ~ oils. Concerning selectivity, silicon oil presented the highest value (~3000), whereas ionic liquids co-extracted important amounts of water and presented the lowest selectivity values (<80). Some positive influence of the branching degree and the position of the polar group into the molecule on the selectivity value was revealed for alcohols and esters investigated.

A biocompatibility screening protocol was developed based on microfermentations, and two parameters were compared to solvent-free control test: glucose consumption and total butanol production (aqueous and organic phases). It was observed that the excess of glucose consumption for biocompatible solvents does not lead systematically to the same amount of overproduction of butanol. In other words, some influence of the solvent used in liquid extraction on ABE metabolism was revealed. Specific experimentation and the study of whole detailed fermentation profiles appear necessary to evaluate these hypotheses and improve comprehension. The solvent 2-butyl-1-octanol presented the best yield for butanol ($Y_{BuOH/Glu}$) and was discovered in this work to have good extraction characteristics. 2-Butyl-1-octanol belongs to the family of Guerbet alcohols and it is commonly used as raw material in the industries of cosmetics, drug delivery, metal processing, fiber finish, thermostable and biodegradable lubricant and solvent, as well as surfactant. However, it has not been extensively used in extractive ABE fermentations, and for this reason a more detailed study needs to be carried out to further evaluate its potential use in butanol production. Other important criteria, like cells viability in a mid-long-term fermentation configuration remained unstudied but should be considered in further steps.

3. Batch extractive fermentation with solvents of different nature

Solvents with opposite characteristics in terms of biocompatibility towards *Clostridium acetobutylicum* and butanol extraction capacity: vegetable oils, 2-butyl-1-octanol (2B1O) and oleyl alcohol were tested in batch extractive fermentations. Concentration profiles of substrate, intermediate and final products were compared with a control (solvent-free) fermentation. By concomitantly removing the inhibitory products, the total initial substrate was completely consumed with both solvents, while about 70% of the substrate was consumed in the solvent-free batch fermentation. Overproduction of butanol attained 60% and 100% with vegetable oil and 2B1O respectively. Surprisingly, in the case of 2B1O extractive fermentation, a 46% increase of butanol yield (mass of butanol per mass of glucose consumed) was also observed, as well as the doubling of the ratio for the main metabolites (butanol to acetone). However, with this solvent, a longer lag phase compared to solvent free or vegetable oil extractive fermentation was observed, although the extraction capacity of butanol improved significantly due to the higher partition coefficient. Based on these results, differences between solvents go beyond what is expected from thermodynamics (liquid-liquid extraction). Culture metabolism and/or membrane polarity related functions appear to be influenced by the presence of 2B1O. In this context, possible mechanisms for these effects are discussed.

Part of this chapter has been redrafted after: Gonzalez-Peñas H., Eibes, G.,Lu-Chau T.A., Moreira M.T., Lema J.M. Altered Clostridia response in extractive ABE fermentation with solvents of different nature. Article under review in *Chemical Engineering Journal*

OUTLINE

3.1 Introduction

Among the different ways of integrating ABE fermentation and liquid extraction, the simplest configuration corresponds to an on-site configuration, where no additional extraction vessel is required. In this case, the solvent is added into the bioreactor, which acts as a one-stage extractor whose operating conditions (agitation rate, temperature, pH) can be varied, but should respect fermentation requirements. In addition, the microorganisms are in direct contact with the extraction solvent, and the possible response of the microorganism to the solvent must be addressed.

Candidate solvents for butanol removal can be defined by their good extraction capacity (distribution coefficient for the products and selectivity) and their biocompatibility. As discussed in Chapter 2, in practice, it is difficult to find a solvent that meets these requirements. For example, partition coefficient and selectivity vary in the opposite direction for most chemical families and biocompatible solvents are generally poor extractants. Solvent toxicity is correlated with their hydrophobic character (less water affinity implies more biocompatibility), and the logP (or $\log K_{octanol\text{-}water}$) value has been used to classify solvents into two subcategories: 1) toxic extractants, those that present values lower than that of a microorganism dependent critical value ($\log P_{critical}$ = 4-5 in the case of Clostridia (Daugulis et al. 1991)), and which are easily disregarded as they prevent any microbial growth and 2) non-toxic solvents, harmless to bacteria, with $\log P > \log P_{critical}$. The latter are supposed to improve not only the titer of the final product but also volumetric productivity, as fewer kinetic inhibitors are present to affect the microorganism. The LogP value is inversely correlated with the alcohol distribution coefficient (Salter et al. 1995) and, consequently, it should be convenient to work as close as possible to the critical logP to increase extraction performance. However, only a few solvents that fall within the “biological threshold” have been tested for butanol or ethanol extractive fermentation and the results have revealed some level of inconsistency (Offeman et al. 2008).

In Chapter 2 of this thesis, a solvent screening for ABE extractive fermentation in small-scale fermentations was carried out. Both extraction capacity (partition coefficient and selectivity) and biological parameters (biocompatibility and bioavailability) were evaluated. However, the high length-to-diameter ratio and the lack of agitation mean that these experiments are not representative of mass

transfer in larger scale configurations such as those typically found in industry, where contact between cells and dissolved solvent extraction molecules would be favored. Solvents considered as toxic in that screening will remain toxic in any configuration, while biocompatible solvents must be evaluated on a larger scale. In addition, the correlation between butanol and butyric acid partition coefficient and butanol yield (mass of butanol per mass of glucose consumed) was a major outcome of that work.

These results led us to further investigate the extractive fermentation of ABE with two selected solvents of opposing characteristics: 2-butyl-1-octanol (2B1O) and vegetable oil. 2B1O is a beta-branched C12 Guerbet fatty alcohol that presents a better partition coefficient for butanol (6.76), but also a higher polarity (log P=4.99). In addition to organic solvents, vegetable oils offer biocompatibility and could represent a green alternative solvent for ABE extractive fermentation, despite their lower separation capacity ($K_{BuOH} < 1$). Pomace olive (PO) oil and sunflower oil (SFO) were selected to be tested for laboratory-scale analysis. Oleyl alcohol (OA), that is often considered as the standard butanol extraction agent in literature since the early 1980s (Roffler et al.1986), was also included in this study for comparison purposes.

The objective of this study is to gain relevant information on solvent-dependent kinetics compared to a solvent-free control system. The runtime profiles of substrate, main intermediates and products in both aqueous and organic phases are essential to understand the phenomena underlying ABE extractive fermentation, so that the most favorable conditions are selected for process scale-up. In this context, the assessment of real performance parameters (yield, productivity) will allow a more realistic techno-economic comparison.

3.2 Materials and methods

3.2.1 Microorganism and culture medium

Clostridium acetobutylicum ATCC824 was used as the solvent producing microorganism. The inoculation procedure of the pre-culture and the composition of the media have been already described (Gonzalez-Peñas et al. 2014).

3.2.2 Control fermentations

Control fermentations were carried out in parallel to in situ two-phase extractive fermentations. These experiments were performed in 250 mL sealed flasks, filled with 100 mL of fermentation medium, and inoculated with 10% (v/v) of cells growing at maximum growth rate. The bioreactor was previously purged with nitrogen for 30 min to guarantee anaerobiosis. The entire system was autoclaved at 120 ºC for 20 min prior to inoculation. Temperature and pH were 34 ºC and 6, respectively. Subsequently, pH varied freely, and samples were withdrawn periodically from the fermentation medium.

3.2.3 Extractive ABE fermentations

Four solvents were tested in biphasic fermentations: two vegetable oils (pomace olive oil and sunflower oil) and two organic compounds (2-butyl-1-octanol and oleyl alcohol, Sigma Aldrich). Some descriptive properties of these solvents are presented in Table 3.1.

Table 3.1. Some properties of the tested solvents

Organic Solvents	*Source*	*Purity%*	*CAS #*	*d (kg/m^3) at 20 °C*	*BP (°C) (P, atm)*	*Viscosity (mPa/s) at 25ºC*
2-butyl-1-octanol	Sigma Aldrich	95	3913-02-8	833	145-149 (0.2)	16.66
Oleyl alcohol	Sigma Aldrich	85	143-28-2	850	330-360 (1)	28.32
Vegetable OIls	*Predominant structure*			*d (kg/m^3) at 20 °C*	*Smoke Point, ºC*	*Viscosity (mPa/s) at 25ºC*
Pomace Oil	Linoleic acid			918	210	26.91
Sunflower Oil	Oleic acid			914	232	26.98

The experiments were carried out in 250 mL sealed-bottle flasks filled with 100 mL of culture medium, following the experimental configuration and methodology described for the control fermentations. For all the experiments, the initial glucose concentration was fixed at 90 g/L to allow the butanol production to increase beyond the inhibition threshold concentration. The temperature was set at 34 ºC and the initial pH at 6. The pH was not controlled through the fermentation, and

the shaker agitation was set low enough (40 rpm) not to break the aqueous-organic interfacial surface of the extractive fermentations. This implies a lower level of emulsion formation -which would facilitate a larger scale operation- and limits the exposure of the cells to the solvent used, reducing the risk of biphasic toxicity. The extractant solvent was added to the flask before nitrogen purge at the beginning of the fermentation. The ratio between solvent and aqueous phases was 1:1 (v/v) and 0.25:1 (v/v) for vegetable and organic solvents respectively. Considering the partition coefficient for the most inhibitory metabolite (n-butanol) in Vegetable Oil (0.62), 2B1O (6.76) and OA (4.57) the specified ratio between phases ensures that the solvent is not saturated before the end of the fermentation, assuming total glucose consumption. The flasks were adapted with a sampling device for both the aqueous and organic phases. The total concentration of each metabolite was calculated as shown in Equation 3.1.

$$[P]_{tot} = (V_{aq}[P]_{aq} + V_{org}[P]_{org})/ V_{aq} \quad (3.1)$$

where $[P]_{tot}$ is the total concentration of the product in relation to the volume of the aqueous phase; $[P]_{aq}$ and $[P]_{org}$, the concentration of the product in aqueous and organic phases, respectively; and V_{aq} and V_{org}, the volumes of organic compounds in the aqueous and organic phases in the bioreactor. Even if the initial ratio of both phases is not maintained (sampled volumes of phases could not be identical), the volume variation of each phase through the fermentation was considered for the mass balances and for the estimation of equilibrium data.

3.2.4 Analytical procedures

Glucose concentration was determined using an enzymatic kit (Spinreact). The aqueous and organic phase compositions were determined by gas chromatography (GC) using an HP6890 GC with N_2 as the carrier gas and a flame ionization detector. The GC was equipped with an Agilent 122-7032E column. The oven was kept isothermally at 70 °C for 3 min and thereafter, temperature was increased up to 200 °C with a slope of 50 °C/min. In the case of extractive fermentation, the metabolites of the organic samples were quantified using two dilution agents: a) hexane, to determine butanol and butyric acid, and b) butanol, to quantify ethanol and acetone. In both cases, hexane and butanol were combined at a ratio of 2:1 (v/v) with the organic aliquot being analyzed. The objective was to ensure a single homogeneous sample at room temperature prior to GC analysis.

3.2.5 Calculations

Process parameters

The product yield of a *P* metabolite is expressed as the ratio between the total product concentration (P_{tot}) and the glucose consumption at the end of the fermentation:

$$Y_{P/Glu} = P_{tot}/(P_{Glu,\,0} - P_{Glu}) \quad (3.2)$$

The overall volumetric production rate of a metabolite *P* ($P_{vol,\,P}$) is calculated taking into account the fermentation time at which the maximum total concentration of the product is attained, including the lag phase, as indicated below:

$$P_{vol,\,P} = P_{tot}/t_{fermentation} \quad (3.3)$$

Overall glucose consumption rate is estimated as described for the metabolites. The instantaneous glucose consumption rate at time t ($P_{vol,\,Glu,\,t}$) is estimated by the concentration variation between two consecutive sample intervals:

$$P_{vol,\,Glu,t} = (P_{Glu,\,t+\Delta t} - P_{Glu,\,t})/\Delta t \quad (3.4)$$

Liquid-Liquid equilibrium approach

Theoretical equilibrium distribution ($P^{eq}_{\ aq}$ and $P^{eq}_{\ org}$) of the total product concentration (P_{ext}) was calculated by mass balance taken into account the partition coefficient of the product *P* (K_P) and the actual volumetric ratio between phases ($r = V_{org} / V_{aq}$) at the calculation time:

$$P^{eq}_{aq} = P_{ext}/(1 + K_P\, r) \quad (3.5)$$

$$P^{eq}_{org} = K_P\, P^{eq}_{\ aq} \quad (3.6)$$

All of partition coefficients used in this work were taken from (Gonzalez-Peñas et al. 2014).

Metabolic analysis

Mass balance analysis was performed based on the theoretical background of ABE fermentation to estimate the relative contribution of the metabolic pathway to butanol formation. Acetone is produced from acetoacetate through an irreversible decarboxylation step, and its formation is linked by the same catalytic enzyme CoA transferase to the acid consumption pathway (Jones and Woods, 1986). Butanol obtained from acetic and butyric uptake was then calculated by molar equivalence from total acetone concentration. To this end, the well-balanced reactions (mass and electrons) of the two acid assimilation pathways proposed by Jones and Woods (1986) were considered, instead of those used by Jang et al. (2012), since electron balance was not included in the latter. The experimental ratio of acetate to butyrate uptake rates (R_{bu}/R_{ac}) obtained in (Jang et al. 2012) by metabolic flux analysis, was applied.

$$R\_ac: 0.5\ Acetate + 1\ Glu \rightarrow 1\ H_2 + 2.5 CO_2 + 0.75\ BuOH + 0.5\ Acetone \quad (3.7)$$

$$R\_bu: 0.5\ Butyrate + 1\ Glu \rightarrow 1\ H_2 + 2.5 CO_2 + 1\ BuOHl + 0.5\ Acetone \quad (3.8)$$

$$R\_ac/R\_bu = 5.5 \quad (3.9)$$

3.3 Results and discussion

3.3.1 Extractive fermentation with vegetable oils

In batch conventional ABE fermentation, glucose utilization is limited by end-product inhibition of *Clostridium* culture. Figure 3.1A shows that extractive fermentation with pomace oil (PO) enhanced glucose consumption from 75% in the free-solvent mode to 100%. Maintaining the toxic product below the concentration threshold in the aqueous phase increased the concentration of total solvents (ABE) from 16 to 28 g/L at the end of the experiment (Figure 3.1A). At the same time, total butanol production increased to 60%, from 10 g/L in conventional fermentation to 16 g/L in the presence of pomace oil (Figure 3.1B). In the extractive fermentation, for comparison purposes, the total ABE concentration (aqueous and organic phases) refers to the volume of the aqueous phase.

Figures 3.1B depicts the evolution of the aqueous and total solvent concentration (butanol, acetone and ethanol) in both control and PO extractive fermentations. In

solvent-free fermentation, the critical aqueous concentration of butanol was reached in 50 h, which corresponds to the moment when metabolic activity was negatively affected, and glucose consumption slowed down (Figure 3.1A). In the extractive mode, the aqueous concentration of products increased until the end of the fermentation, when sugar was no longer available. At that time, the concentration of aqueous butanol reached the inhibition threshold (10.75 g/L), indicating that the capacity of the solvent extraction has been fully exploited, and to extract more butanol, oil regeneration would be needed. Figure 3.1B indicates that the concentrations of aqueous acetone and butanol approach the end of extractive fermentation (9.42 and 10.75 g/L, respectively). In fact, the relative partition coefficients between metabolites (0.62 and 0.39 for butanol and acetone respectively) will determine the final composition of the broth and could influence the downstream separation. In the PO extractive fermentation, both aqueous and total butanol concentrations increased until the end of the experiment (Figure 3.1B). However, the global butanol productivity was similar in both configurations, indicating that the removal of toxic product achieved with PO extraction allowed for longer and more effective fermentation without significantly enhancing fermentation kinetics.

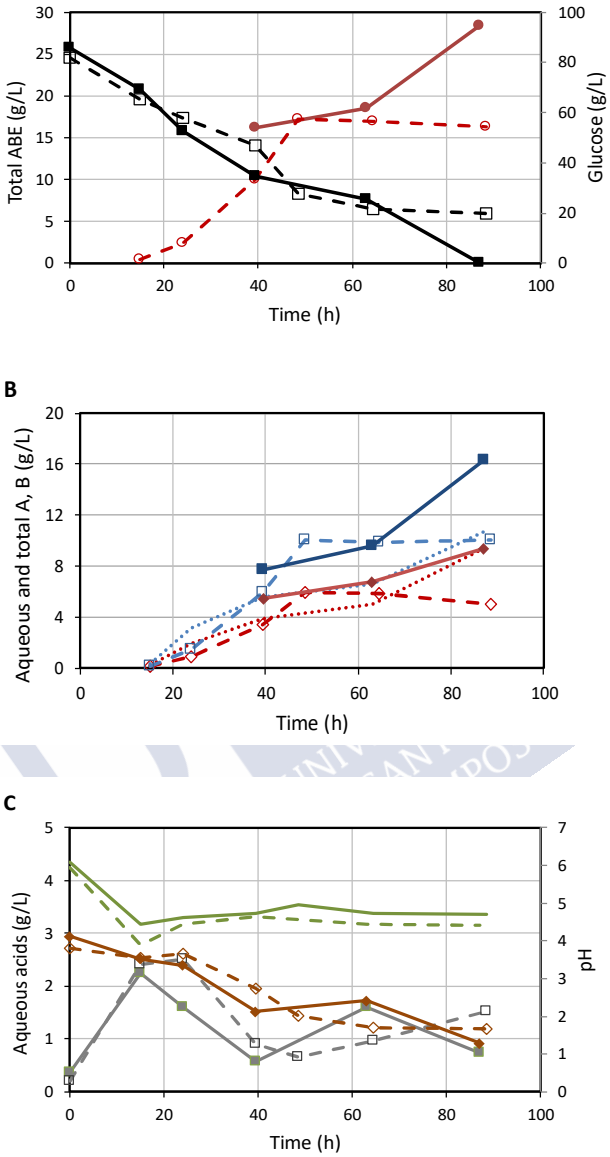


Figure 3.1. Control (dashed line) and extractive fermentation with PO (continuous and dotted line for total and aqueous values respectively). A) Profiles of glucose (square) and total ABE concentration (circle). B) Profiles of butanol (square) and acetone (rhombus) C) Profiles of pH (no marker), aqueous butyric (square) and acetic (rhombus)

During the control fermentation, butyric acid was produced in the acidogenic phase up to 2.5 g/L before being assimilated to butanol (from the metabolic switch at about 20 h of fermentation, Figure 3.1C). The pH profiles showed the typical time course of ABE fermentations in both control and extractive configurations (Fig 3.1B). The profiles of the aqueous concentration of butyric and acetic acid were similar in the control and PO extractive fermentations during the first part of the experiment. In fact, the concomitant acid co-extraction during acidogenic phase was limited with vegetable oil due to the low partition coefficient ($K_{butyric}$ = 0.22). Nevertheless, some differences were observed during the solventogenic phase. In the control fermentation, butyric acid decreased after the metabolic switch (around 20 h), and increased again after 45 h. At that time, glucose was still available, even if butanol had reached its maximal value. In the case of the extractive fermentation, two clear consecutive cycles of acidogenic-solventogenic phases were achieved, as seen in Figure 3.1C. Therefore, butyric acid was assimilated into butanol between 60 and 80 h of fermentation, according to Figure 3.1B. Interestingly, solvent productivity rate at the end of the fermentation was as high as at the beginning (Figure 3.1B), despite that the butanol concentration in the aqueous phase was higher than 8 g/L.

Table 3.2 compares the main performance parameters obtained for control fermentation and for both pomace and sunflower oil (PO and SFO, respectively). The two vegetable oils performed similarly due to their structure, mainly dominated by fatty acids. Butanol and ABE yields slightly improved in PO extractive fermentation (16% and 24%, respectively) according to the results presented in (Gonzalez-Peñas et al. 2014).

The application of vegetable oils as solvents in ABE extractive fermentation has already been reported. Ishizaki et al. (1999) compared an extractive fermentation of methylated crude palm oil with solvent-free fermentation, both with 90 g/L of initial glucose. Sugar consumption increased from 60 to 83% in the extractive fermentation, which is comparable with the results obtained in this study (Table 3.1). In that study, 47% of total butanol was extracted, while in the present study 34% of the butanol was eliminated. This is also consistent with the differences in the extraction capacity of the solvents used (K_{BuOH} = 0.6 for PO and K_{BuOH} = 0.9 for crude palm oil). Li et al. (2010) reported improved butanol and ABE production (42% and 50%, respectively) using biodiesel in extractive batch fermentation of ABE,

when the solvent was optimally added after 48 hours of fermentation. Butanol overproduction was higher in this work (60%), in spite of the greater solvent capacity of biodiesel (K_{BuOH} >1). In both references, the authors pointed out the possibility of the direct use of the alcohol-enriched vegetable oil in fuel applications, since intrinsic properties are improved with alcohol extraction, thus avoiding the regeneration related cost. Castor oil was also tested (Gonzalez-Peñas et. al 2014), but its lower selectivity and high viscosity make its use less attractive for commercial purposes, despite its higher butanol partition coefficient (K_{BuOH} = 2.6). Moreover, the toxicity of ricinoleic acid (the main constituent of castor oil) for *Clostridium acetobutylicum* was previously proved (Ishii et al. 1985).

3.3.2 Extractive fermentation with organic solvents

Extractive fermentation with 2B1O showed significant variability in time profiles between replicates (flasks A and B). However, important similarities in their behaviour on control fermentation deserve to be highlighted. The variability in results obtained with solvents that are at the limit of toxicity towards Clostridia in terms of logP value, as in the case of 2B1O, has been previously reported (Offeman et al. 2008).

Glucose consumption increased above the control fermentation in both duplicates, but only in one flask glucose was depleted. While sugar over consumption was 53 and 19 % , total ABE production was increased by 84 and 60% in flasks A and B, respectively (Figures 3.2 A-D). Nevertheless, in both 2B1O extractive fermentations the metabolic activity (glucose consumption and solvent production) was delayed with respect to the control (Figure 3.2). After 30 h, 90% of the solvent production was achieved in the control fermentation, while 2B1O extractive fermentations had just started the solventogenic phase.

Figures 3.2B and 3.2E show the evolution of the total and aqueous concentration in the control and the 2B1O extractive fermentations. In the control fermentation, the critical aqueous concentration of butanol was reached around 45 h, when glucose consumption ceased. In the extraction experiments, the aqueous concentration of butanol remained low (< 6-8 g/L) throughout fermentation, which is explained by the high partition coefficient of butanol in this solvent (6.76). As a result, up to 64% and 72% of the total butanol produced was transferred to the organic phase in flasks A and B, respectively, while acetone and

ethanol concentration in the aqueous phase remained above 80 and 90% in the two replicates.

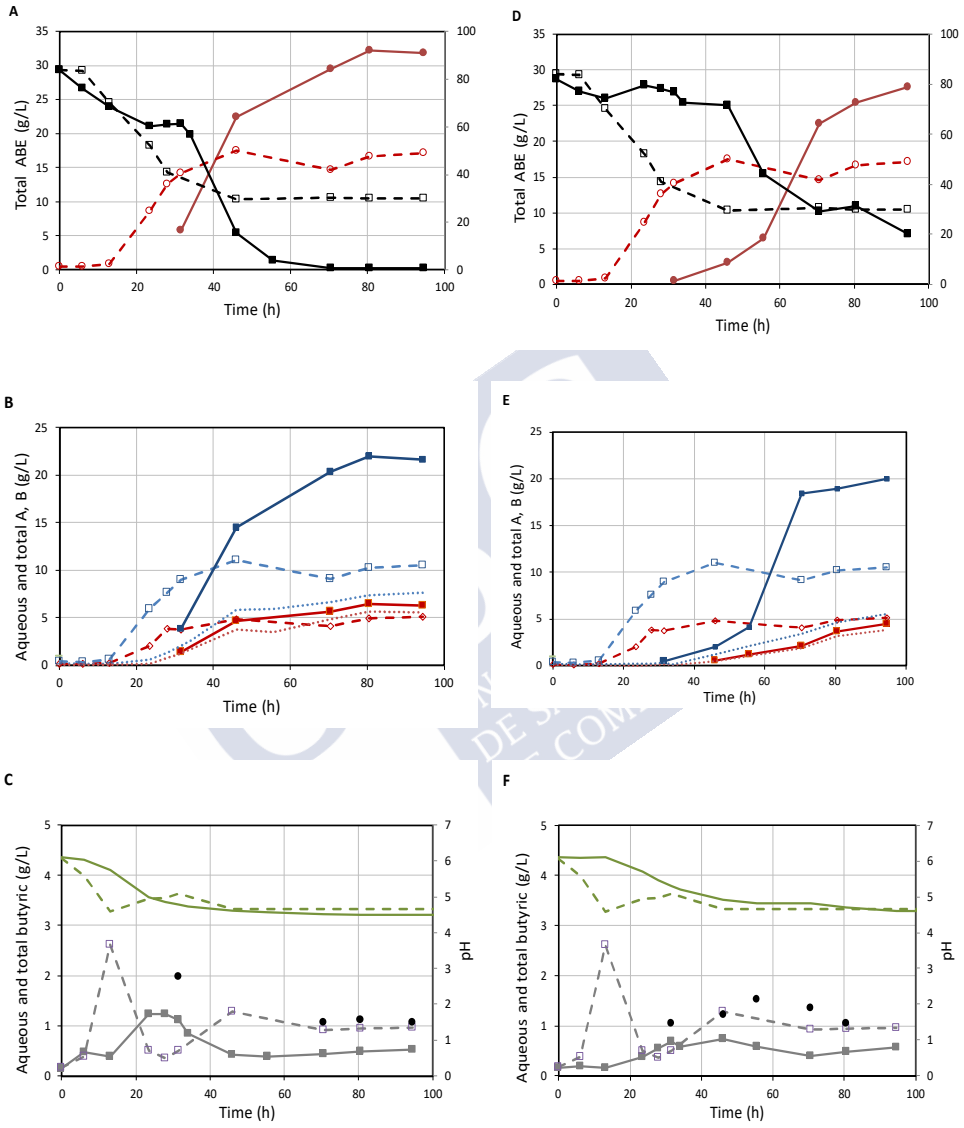


Figure 3.2. Control (dashed line) and extractive fermentation with 2B1O (continuous and dotted line for total and aqueous concentration respectively) in duplicate flasks cultures: A (left) and B (right). A) and D) Profiles of glucose (square) and total ABE concentration (circle) B) and E) Profiles of butanol (square), acetone (rhombus) C) and F) Profiles of pH (no marker), aqueous butyric (grey square) and total butyric (black circle)

2B1O extractive fermentations showed an atypical pH trend (Figure 3.2C and 3.2F). In both flasks, pH decreased from an initial value and then stabilized at around 4.8. The typical “pH switch”, reflecting the Clostridia metabolic transition from acidogenic to solvent stages in conventional batch ABE fermentations, was not observed in the 2B1O extractive fermentations. Partition coefficient for butyric acid in 2B1O is high (6.96), so a significant fraction of the total butyric acid produced in the aqueous phase was transferred to the organic phase during acidogenesis. This can be seen in Figure 3.2, which shows both aqueous and total butyric acid concentrations. Consequently, to maintain equilibrium after the metabolic shift, the butyric acid was transferred back to the aqueous phase while the microorganism converted it to butanol. Accordingly, the organic phase may act as a butyric acid reservoir during the acidogenic step to be concomitantly co-fed with glucose during solventogenesis, so there is no external pH variability.

The maximum glucose uptake rate started only after 30 and 40 h for 2B1O extractive fermentations in flasks A and B, respectively. Considering these lag times, the overall sugar consumption rate in flask A was similar to that of the control, while it slightly decreased in flask B (Table 3.2). However, as shown in Figure 3.3, the maximum glucose consumption rates were reached in the 2B1O extractive fermentations between 40 and 50 h (3.5 and 3 g glucose/(L·h) for flasks A and B respectively), while the maximum rate in the control culture (2.5 g/(L·h)) was reached after 30 h. The total solvent production rate in the extractive fermentations also enhanced in the same period (data not shown), indicating that the solventogenic capability was maintained in the presence of 2B1O, although a longer adaptation time was required.

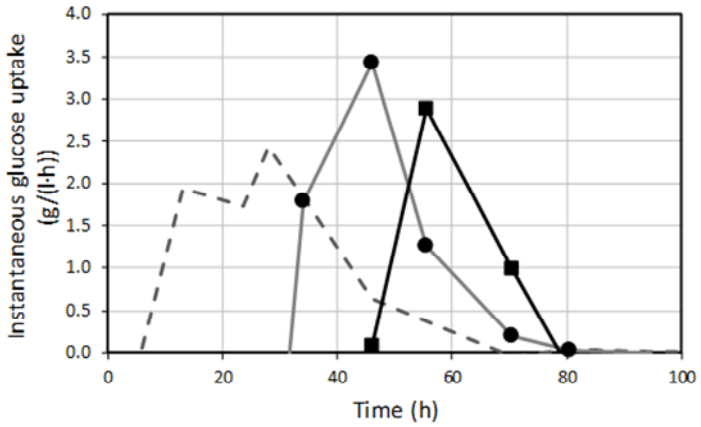


Figure 3.3. Profile of glucose uptake rate during batch fermentations. Control (dashed line) and extractive (continuous line) fermentation with 2B1O in duplicate flasks cultures a (circle) and b (square).

Table 3.2 summarizes the results obtained in the control and extractive fermentations. Although showing similar trends, the quantitative results of the time-dependent variables in both duplicates were different. Similar inconsistencies were observed in previous works during the extractive fermentations with the solvents placed in the "critical region" in terms of polarity or logP. These solvents are typically C11-C14 compounds, such as 2-butyl-1-octanol. Offeman et al. (2008) used dodecanol as a solvent in ethanol extractive fermentations and found that the results were not repeatable and very sensitive to test conditions.

Oleyl alcohol (OA), which is the reference in ABE extractive fermentation, was also evaluated in the same operating conditions as 2B1O. With OA, 76 g/L of glucose were consumed (instead of 51 g/L in the free-solvent control), but the concentration of butanol in the aqueous phase reached the inhibition threshold at the end of the fermentation (10.75 g/L). Butanol production increased by only 31% compared to the control experiment.

Tabla 3.2. Summary of results

		Vegetable Oils		*Organic Solvents*		
	Control (SD)	*PO*	*SFO*	*2B1O-1*	*2B1O-2*	*OA*
*Glucose uptake rate, g/(L·h) **	1.08 (0.15)	0.98	0.95	1.20	0.69	0.98
*Butanol productivity, g(L·h) **	0.21 (0.03)	0.19	0.18	0.27	0.21	0.19
Butanol, g/L	10.25 (0.35)	16.30	15.50	21.40	20.03	17.95
Acetone, g/L	5.01 (0.44)	9.36	9.02	6.18	4.44	6.07
Ethanol, g/L	1.40 (0.23)	2.65	3.56	4.02	3.10	4.65
Butanol yield, g/g	0.18 (0.02)	0.19	0.19	0.25	0.31	0.24
ABE yield, g/g	0.29 (0.04)	0.33	0.34	0.38	0.43	0.38

(*) Time dependent variables calculated considering final values and including lag time

3.3.3 Acetone-Butanol (AB) ratio

In Chapter 2, a higher yield of butanol was obtained over control, in extractive fermentation with organic solvents presenting high affinity for butanol and butyric acid. This result is corroborated by the differences in the yield of the final product obtained with organic solvents. As shown in Table 3.2, both butanol and total ABE yield are improved with 2B1O compared to solvent-free base case, but butanol yield improvement is at least 65% higher. It is therefore expected that the improvement in butanol production compared to the glucose consumed would be somewhat offset by the decrease in co-product yield. This is verified in Figure 3.4, where the mean values of the butanol/acetone ratio are plotted along with the mean experimental yields of butanol and ABE, for the control and solvent extractive fermentations.

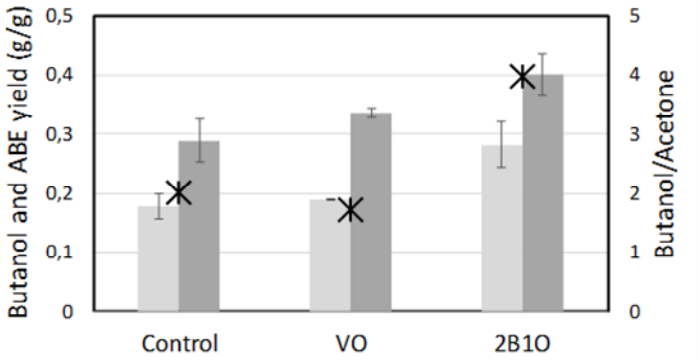
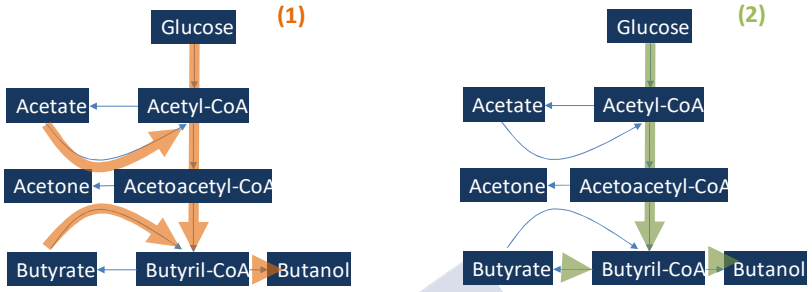


Figure 3.4. Average yield values of butanol (light grey) and ABE (dark grey), and butanol/acetone ratio (asterisk) found in control and extractive fermentations.

There are at least two possible metabolic pathways for butanol formation during ABE solventogenesis: 1) classical acid uptake pathway (butyric and acetic) and 2) direct pathway from acetyl-CoA to butyril-CoA condensation, and its subsequent reduction to butanol (Jang et al 2012). The former option (acid assimilation) is catalyzed by the enzyme CoA transferase (CoAT) and is concomitant to irreversible production of acetone and CO_2, which is reduced the final yield of butanol. In Figure 3.4, the butanol/acetone ratio obtained in 2B1O extractive fermentation doubled that of VO or control fermentation. Table 3.3 includes estimates of the relative contribution to butanol production of both metabolic pathways for the results obtained in this work, considering the total molar acetone equivalent. In fact, during acid assimilation, 0.5 mol of acetate would result in the formation of 0.5 mol of butanol and 0.5 mol of acetone, whereas uptake of 0.5 mol of butyrate would give 0.5 mol and 0.75 mol of acetone and butanol, respectively (Jones and Woods 1986). The ratio of the rates of both acid uptake (acetic/butyric uptake) was taken from (Jang et al. 2012) for these calculations.

Table 3.3. Estimation of the relative contribution of butanol metabolic pathway by molar equivalence. Orange arrows in metabolic pathway (1) highlight the classical acid uptake pathway and green arrows in (2) represent other metabolic possibilities



	B from 1, mM	*%B from 1*	*B from 2, mM*	*% B from 2*
Control	102.6±8.5	72.0	39.8±4.0	28.0
VO	175.7±4.6	79.5	45.26±3.3	20.5
2B1O	*101.5±23.5*	*35.1*	*186.26±10.0*	*64.9*

The percentage of butanol formation by the acid conversion pathway decreased by half in the case of 2B1O extractive fermentation compared to control or vegetable-oil based fermentation, which explains the increase in butanol yield obtained with this solvent. To our knowledge, the response of metabolism in extractive fermentation in favor of butanol yield by increasing the relative contribution of the direct formation pathway of butanol metabolism has not been previously reported. However, it is necessary to clarify the reasons for this performance improvement.

The increase in butanol yield in 2B1O extractive fermentation could be explained by the additional transformation of butyric acid from the organic phase, which was transferred back to the aqueous phase during solventogenesis. Supplementation of a solventogenic culture with butyrate has been reported to improve glucose butanol yield (Tashiro et al. 2007, Lee et al. 2008). However, this effect was not observed in previous extractive fermentation with OA, despite its significant butyric extraction capacity ($K_{butyric}$ = 1.85). We hypothesize that the solvent did not act as a

butyric reservoir in those cases, because it was added either after the acidogenic phase (Ishizaki et al. 1999) or once the aqueous inhibitory concentration of butanol in the fermentation broth was reached (Ishii et al. 1985).

A complementary hypothesis to explain the increase in butanol yield is that butyrate conversion may be partially mediated by the inverse reaction between butyril-coA and butyrate (Lehman et al. 2012) (Dessai et al. 1999). Therefore, it would not necessarily be associated with acetone formation and the requirement of the CoAT enzyme. This would involve the participation of the enzyme butyryl-P in the assimilation of butyric acid into butanol. It has been reported that this enzyme is sensitive to ΔpH in the pH range of *Clostridium* fermentation (Evans & Wang 1990), which means that the addition of acids or other uncouplers may affect acid production and absorption. In fact, the effects of ΔpH on the production/consumption of butyrate are supported by our experimental data. Figure 3.5 shows the profile of undissociated aqueous butyric acid through control and extractive fermentation with 2B1O. In the presence of 2B1O, solventogenesis was effectively and fully achieved, while the concentration of undissociated acids remained below the expected activation threshold in the fermentation medium.

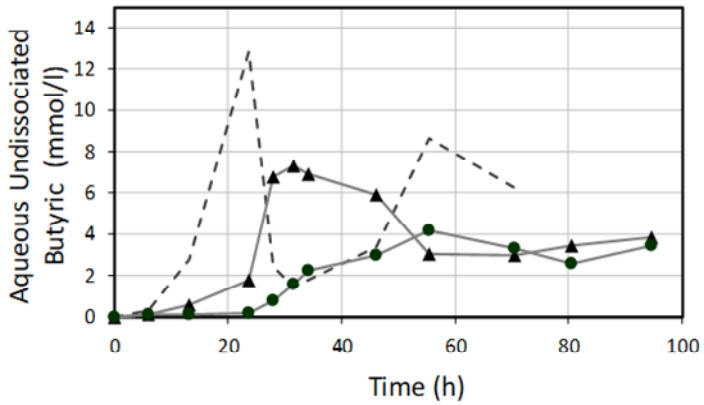


Figure 3.5. Profile of aqueous undissociated butyric acid during batch fermentations. Control (dashed line) and extractive (continuous line) fermentation with 2B1O in duplicate flasks a (triangle) and b (circle).

It is assumed that a minimum intracellular concentration of undissociated acid is necessary to trigger solventogenesis (Jarzebski et al. 1992) (Monot et al. (1984). Terraciano and Kashket (1986) stated that solvent production starts at an undissociated butyric acid concentration of 13-14 mM in the aqueous broth. As the undissociated acid pass freely inside the cells, both external and internal pH

decrease during the acidogenic phase, while ΔpH remains constant across the cell membrane by the action of membrane-bound ATPase (Monot et al. 1984, Terraciano and Kashket 1986). Solvent production is induced to circumvent a decrease in intracellular pH large enough to be detrimental to the microorganism (Jones and Woods 1986). However, if the harmful effect of weak acids leads to acidification within the cell, the solvent (butanol) causes chaotropic effects on the membrane. The inhibition of ATPase is one of the reported toxic effects (Tomas et al. 2004). As a result, the ability to maintain the ΔpH across the cell membrane is subject to the stress imposed by the fermentation products. The decrease in ΔpH leads to the increase of the concentration of undissociated acids within the cell, since the dissociation equilibrium is modified. Dissolved molecules of 2B1O may cause the same effect as butanol, since linear alcohols have been reported to have a similar effect on the microorganism (Ingram & Buttke 1984). Therefore, even with a low total butyric acid concentration in the aqueous medium (Figure 3.5), 2B1O in the aqueous phase could increase the undissociated acid concentration inside the cell, and thus trigger solvent production. The positive influence on butanol production by lowering intracellular pH by inhibition of ATPase was observed by (Monot et al. 1984). Evans and Wang (1988) observed the increase in butanol production when 20% of decanol was added to oleyl alcohol in batch ABE extractive fermentation. Physical extraction alone could not explain this increase, and it was hypothesized that dissolved decanol would influence metabolism under certain conditions.

Cell agglomeration

Interestingly, cells agglomeration was visually appreciated during the two 2B1O extractive fermentations. A clear formation of pellets is progressively decanted during the early stages of fermentation (Figure 3.6). Neither solvent-free control fermentation, nor extractive fermentation with vegetable oils or OA presented this phenomenon.

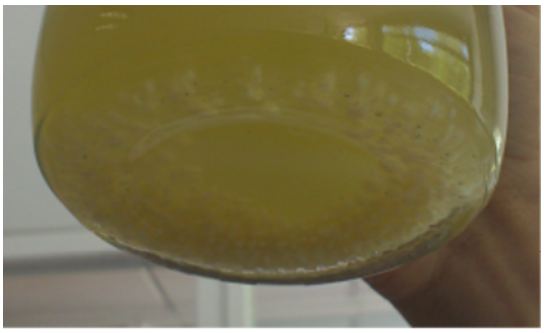


Figure 3.6. Formation of cell aggregates in 2B1O fermentation

It has been reported that natural cell self-aggregation is an indirect approach to improve the performance of Clostridia cultures in bioreactors by increasing cell density and protecting against hostile environments or stress (Pugazhendhi et al. 2018) (Liu et al. 2018). It has been proposed that the release of extracellular polymeric substance (EPS) is key to granule formation (Craczyk & Myszka 2007). Papers have been published on the capacity to generate EPS and the formation of granules that promote self-flocculation by some clostridial species such as *C. pasteranium* (Liang et al. 2010). These authors induced rapid granulation in Clostridia-enriched mixed culture by combining thermal shock and acidic conditions.

Butanol toxicity has also been identified as a factor triggering the release of cell-free autolysis in ABE fermentation (Barber et al. 1979). Although the concentration of aqueous butanol in this study was always below toxicity levels, it is known that aliphatic alcohols resulted in similar membrane-related toxicity (Ingram & Buttke 1984, Jones & Woods 1986). Accordingly, few dissolved 2B1O molecules could provoke a response similar to that caused by an inhibitory titer of butanol by increasing autolysis production at an early stage of fermentation, forcing Clostridia cells to agglomerate. For this reason, the toxicity of 2B1O should be taken into account when extrapolating the results from flask to bioreactor, since the latter usually presents a lower L/D ratio, more favorable conditions for liquid-liquid transfer, and a more effective mechanical agitation. Under these conditions, dissolved toxicity effects may appear earlier, as 2B1O can more easily be transferred to the aqueous phase.

Mass-transfer between phases

The identification of mass transfer limitations in a two-phase bioreactor is indispensable for optimal reactor design and successful scale-up. Liquid-phase bioprocesses suffer the presence of surface-active materials, which can significantly impact interaction between both phases (Pursell et al 2004). On the other hand, operating conditions favoring mass transfer imply high agitation or emulsion formation (organic droplets) to enhance the available liquid-liquid surface. Figure 3.7 shows that even though a low agitation was applied to not break the interface between phases, butanol transfer was not limited at the flask scale extractive fermentations with vegetable oil and 2B1O. The same conclusion was obtained for butyric acid in the extractive fermentations with the two type of solvents (data not shown). With OA, Figure 3.7 shows that aqueous and organic butanol concentrations do not fit with equilibrium trends estimated with the partition coefficient obtained in Chapter (K=4.57 %wt). These differences might rely on solvent high viscosity at fermentation conditions, which affect negatively mass transfer between phases.

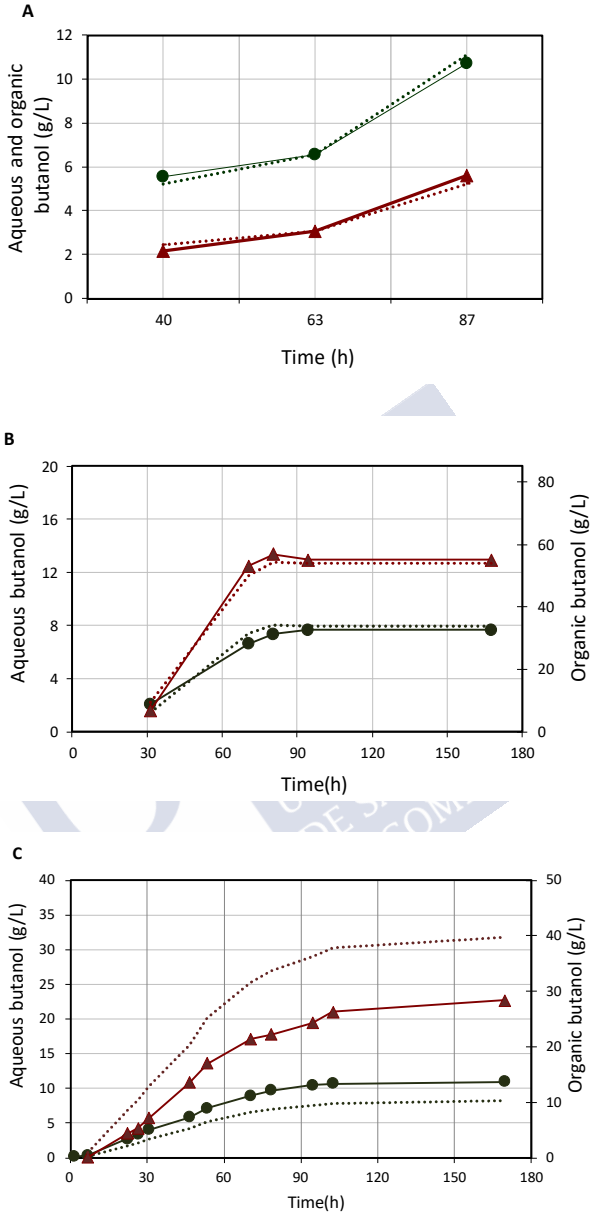


Figure 3.7. Profiles of aqueous (circle) and organic (triangle) butanol concentration for VO (A), 2B1O (B) and OA (C) extractive fermentations. Corresponding equilibria concentration is shown as a dotted line.

Greater mass transfer in a two-phase reactor can also have negative consequences when it comes to polar solvents, as seen in this paper. Further agitation could accelerate toxicity by increasing the presence of toxic extractive solvent droplets in the aqueous phase, thus improving the contact interface and increasing biphasic toxicity (Bruce & Daugulis 1991). Agitation in the experimental conditions evaluated in the present study was not necessary to ensure transfer of metabolites between phases. In larger scale reactors, the geometry will be more favourable for mass transfer (smaller L/D, larger exchange area). However, mechanical agitation may be necessary to achieve a complete suspension of the biomass. If low mechanical agitation is required in the two-phase reactor, and both phases can be kept clearly separate, continuous on-site regeneration of the solvent may be easier.

3.4 Conclusions

The *in situ* extraction of butanol from fermentation media with selected solvents of different nature, a fatty Guerbet alcohol (2-butyl-1-octanol), oleyl alcohol and vegetable oils (pomace olive oil and sunflower oil), was tested on laboratory scale in biphasic sealed flask experiments. By removing the inhibitors from the diluted broth, the initial substrate was fully consumed after the extractive fermentations of 2B1O and pomace oil, while in the solvent-free control a consumption of about 70% was observed. In the extractive fermentations with two solvents of different nature (pomace oil and 2-butyl-1-octanol, respectively) an overproduction of butanol of 60 and 100% was reached. In the case of 2B1O extractive fermentation, there was also a significant increase in butanol yield (46%), and it was found that the ratio of two main metabolites (butanol to acetone) doubled. However, with this solvent, a longer latency phase was observed compared to extractive fermentation without solvents or with vegetable oil. The differences observed between solvents go beyond what is expected from the thermodynamics of liquid-liquid extraction. Culture metabolism and some membrane-related functions also appear to be influenced by the low biocompatibility of 2B1O, and the possible mechanism of these effects were discussed. The investigation of the ABE extractive fermentation response at the microbial population level will be the object of the next chapter of this work.

4. Microbial population dynamics

ABE fermentation by *Clostridium acetobutylicum* was investigated in extractive fed-batch mode through 2-L stirred tank bioreactor experiments. In conventional fermentations, metabolic activity ceases when a critical threshold products concentration is reached (~21.6 g solvents/L). Solvents production was increased up to 36.6 and 37.2 g/L, respectively, using 2-butyl-1-octanol (aqueous to organic ratio 1:0.25 v/v) and pomace olive oil (1:1 v/v) as extraction solvents. The morphological changes of different cell types were monitored and quantified using flow cytometry. Butanol production in extractive fermentations with pomace olive oil was achieved mainly by vegetative cells, whereas the percentage of sporulating cells was lower than 10%.

Part of this chapter has been redrafted after: Gonzalez-Peñas H., Lu-Chau T.A., Moreira M.T., Lema J.M. 2015. Assessment of *Clostridium acetobutylicum* morphological changes in ABE extractive fermentation by Flow Cytometry. Biotechnology Letters, 37: 577-584.
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OUTLINE

4.1 Introduction

ABE fermentation by Clostridia microorganisms is a complex process that involves at least two phases: acidogenic (growth associated production of carboxylic acids with a consequent decrease of pH) and solventogenic (assimilation of acids into solvents, during a pseudo stationary phase) (Lütke-Everlosh and Bahl 2011). The enzymes involved in solventogenesis are triggered by several factors including external pH and intracellular undissociated acids. The metabolic switch that is typically observed before the solventogenesis has also been considered as an adaptative response of the cells to the low intracellular pH (Jones & Woods, 1986). In parallel to these metabolic changes, the solventogenic *Clostridia* used in ABE fermentations goes through a complex cell-cycle during butanol production. During acid production (exponential microbial growth), the so called "vegetative cells" are predominant. They are highly motile and rod shaped. When solvent production begins, cells evolve into fattened cigar-shaped "clostridial cells". Some of them will then sporulate; the free spores ejected into the broth have no metabolic activity but could germinate if conditions are adequate (Schuster et al. 1998). Therefore, distinct subpopulations of the microbial culture coexist in the bioreactor during ABE fermentation, each one with a specific phenotype and metabolic activity associated. The role of the different coexisting cell types has been discussed in literature (Schuster et al. 1998, Tracy et al. 2008), although a simple connection between cell-types and fermentation product has not been yet established.

Deep understanding of the metabolism is essential to develop an industrial process. It allows either to monitor and properly design an advanced process control of the system or can support modeling and biocatalyst improvement. The clostridia cell-cycle and the triggering of solventogenesis (metabolic switch) are often adopted as the basis for investigating the biology around product formation in batch ABE fermentation. The application of quantitative approaches to map the culture heterogeneity in the bioreactor would allow to explain and predict the observed oscillations in continuous culture (Ennis & Maddox 1989).

To investigate the relationship between clostridia cell cycle and metabolites formation rate, and the effect of cultivation conditions on triggering the metabolic switch, it is necessary to identify adequate analytical tools for monitoring the phenotypes evolution during ABE fermentation. The most commonly used method to follow solvent production is gas chromatography. The research of alternative and simpler, more easily implementable on-line cell-cycle markers for ABE product formation has been the object of several works. Schuster et al. (1998) carried out experiments to distinguish acidogenic and solventogenic phases and proposed some direct markers to determine the status of a batch fermentation. Among them,

the alkali consumption (assuming fixed pH) allows to quantitatively follow acid production (and thus biomass growth), while gas composition ratio (hydrogen to carbon dioxide) is a qualitative indicator of the metabolic phase in batch fermentation (ratio <1 or >1 for solvent and acid production phases, respectively). The degree of polarizability of Clostridium culture during fixed pH batch fermentations was determined by electrooptical measurement and was used by Junne et al. (2007) to quantify the mean cell length and to monitor metabolic switch. The stress suffered by the cells while triggering the solventogenic phase resulted in a decreased cell polarizability. However, single cell techniques provide the best and, in some cases, the only means to characterize the heterogeneity of a population (Shapiro 2000). Schuster et al. (1999) applied FTIR (Fourier Transform Infrared Spectroscopy) to Clostridia cultures corresponding to different times of batch fermentations revealing pronounced differences in morphology and chemical composition of the cells. Shuster, et al. (2000) reported the use of Raman spectroscopy to characterize bacteria population in ABE fermentation by single cell analysis. Although powerful, these techniques do not allow to high throughput analysis because they are not capable of quantify bulk population heterogeneity, often require custom-made equipment or long time of sample preparation and analysis (Tracy et al. 2008).

Over the last decades, flow cytometry (FC) has evolved as a powerful tool to qualitative and quantitative assess the biological and physical characteristics of individual cells (Lopes da Silva et al. 2012, Diaz et al. 2010). Flow cytometry (FC) enables to analyze the evolution of bacterial morphology at the single cell level (size and internal granulometry). Combining this technique with specific fluorescent stains, it is also possible to discriminate cells in different physiological states and metabolic activities (Tracy et al. 2010).

Flow cytometry is a flow-based technique allowing to characterize a population of cells or particles by the simultaneous measurement of their multiple physical, chemical or biological characteristics (Tracy et al. 2010). The sample containing the suspension of cells is injected into the flow cytometer (Figure 4.1) as a single stream of particles in the flow cell and pass through a laser beam. The scattered light is received by the photoreceptors and is translated into valuable information about the individual cell geometry and their components. Scattered and fluorescence emissions of each particle are separated by filters and mirrors (optical system) according to certain wavelengths (Diaz et al. 2010). Tens of thousands of cells can be quickly examined, and the data gathered are processed by a computer.

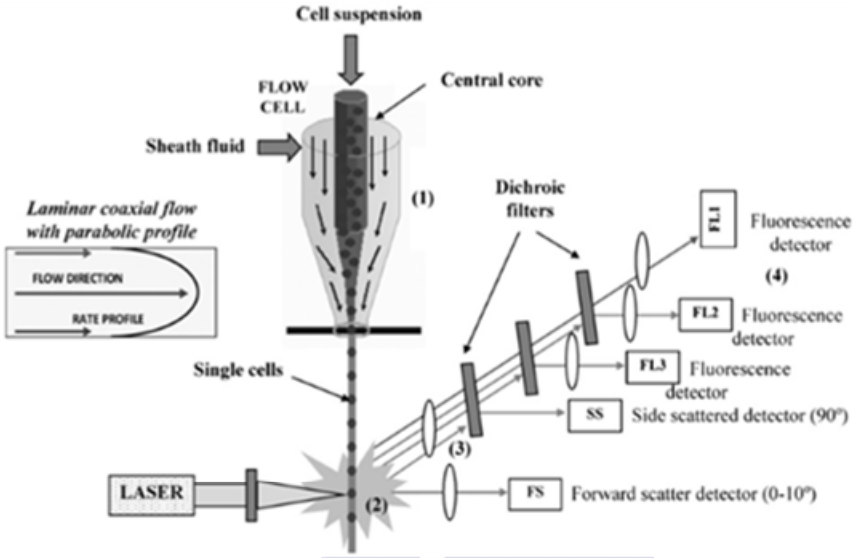


Figure 4.1 Flow cytometer set up (from Diaz et al. 2010)

Therefore, it is possible to collect several types of information:

o Forward scatter (FSC): it depends on the light intensity received by a photoreceptor placed in the same axis of the laser. This signal is related to the diffraction cone, which is function of the cell size. The FSC allows to estimate the cell size.

o Side scatter (SSC): it depends on the intensity of the refracted light, received by a photoreceptor placed perpendicular to the laser beam. The intensity of the diffracted light is proportional to the internal complexity of the cell. The SSC is an estimator of granulometry or internal complexity of the cell.

o Fluorescence (FL): Cells can be stained with one or more fluorescent markers so that light is first absorbed and then emitted in a band of wavelengths to several fluorescence detectors. FL provides supplementary cell information depending on specific used stain.

Finally, signals are sent to a computer obtaining a representation of the distribution of the population with respect to the different parameters. (Diaz et al. 2010). Although microbial flow-cytometry analysis has been advancing for more than 60 years now, it is employed to a very low extent for microbial compared to mammalian cell culture analysis (Tracy et al. 2010).

There have been several attempts to monitor the heterogeneity at the single cell level in ABE fermentation, and to link the production rate of the metabolic products with the physiological state of the culture. Tracy et al. (2008) reported the

first application of flow cytometry to study the cell cycle of *Clostridium acetobutylicum* ATCC824. Combining light scattering with staining and cell sorting protocols, they could discriminate, quantify and enrich all sporulation related subpopulations. The evolution of FSC/SSC signal profile was observed during batch fermentation, and, with the help of phase-contrast optical microscopy, four subfamilies were distinguished, from soon vegetative cells to free spores. The authors concluded that FSC/SSC signal increased during fermentation due to sporulation. Relating these observations with measured butanol flux rate in a batch fermentation, the classical association of the clostridia cell as the solvent-formation phenotype was challenged. Tracy et al. (2008) differentiated the phases of the cell cycle by double staining with Syto 9/PI. It had been previously observed that Clostridia behave in unpredictable ways with certain fluorescent markers traditionally used to assess cell viability in bacteria. Instead, these markers can be used to referee physiological changes in *C. acetobutylicum* (Jones et al. 2008). In the case of sporulating *C. acetobutylicum*, PI stained not only dead cells, but also cells in vegetative state and in early stages of sporulation. However, more recent studies showed that this specific behavior towards PI stain is not necessarily systematic for all solventogenic Clostridia. Linhova et al. (2010) observed a significant thinning of the peptidoglycan layer of *Clostridia* as a result of the onset of solvent production. By the use of an alternative gram double stain Syto 13/HI on *C. pasteranium*, the authors pointed out that vegetative cells are $Gram^{+}$, while solventogenic cells showed a $Gram^{-}$ behavior. Linhova et al. (2012) successfully applied PI to assay the viability of two solventogenic clostridial species (*C. beijerinckii* and *C. pasteranium*). Their protocol was based on the quantitative FC data comparison between exponential growth phase and heat-shocked (dead) cells. Based on double staining with CFDA/PI Kolek et al. (2016) developed a one-step method to referee free spores. This methodology was applied to sporulating and non-sporulating Clostridia by Branska et al. (2018). In that work, the viability response towards changes in cultivation conditions of the batch culture (pH, butanol concentration) was evaluated. A systematic decline in viability was observed after the pH switch independently of the butanol titer in the broth. It was concluded that a) solvent production is not conditional on sporulation, in agreement with Tracy et al. (2008), and b) changes in viability are not directly linked to toxic metabolite accumulation in the broth but might instead be associated with a cell survival strategy triggered by the pH shift.

In the present study, we have assessed the morphology changes of *Clostridium acetobutylicum* by means of FC during liquid-liquid ABE extractive fermentations using two solvents (pomace olive oil and 2-butyl-1-octanol) with different levels of biocompatibility towards *C. acetobutylicum*. Also, membrane permeability was determined by analyzing the cells stained with propidium iodide (PI). In Chapters 2

and 3, it was observed that Clostridia metabolism was influenced by the presence of 2B1O. Therefore, in the case of extractive fermentations, whith additional solvent- cell metabolism interactions, and long-term biocompatibility must be guaranteed, the quick assessment of the physiological status of the culture might be useful for the process development.

4.2 Materials and methods

4.2.1 Microorganism and culture media

Clostridium acetobutylicum ATCC-824 was selected as model microorganism. All experiments started from a frozen spore suspension in saline solution. Spores (150 µL) were heat-shocked for 1 min at 100 °C to induce germination. Then they were used to inoculate 10 mL of potato/glucose preculture medium, which was incubated anaerobically for 72 h at 36 °C. The composition of the preculture medium per liter was as follows: 250 g potato, 2 g $(NH_4)_2SO_4$, 2 g $CaCO_3$ and 10 g glucose.

The pre-culture medium was transferred as inoculum to 100 mL of culture medium in a 250 mL sealed-bottle flask, which was previously purged with nitrogen to guarantee anaerobiosis. The culture medium composition per liter was as follows: 6.6 mg $FeSO_4x7H_2O$, 0.56 g $MgSO_4x1H_2O$, 1 g KH_2PO_4, 0.6 g K_2HPO_4, 2.9 g CH_3COONH_4, 0.1 g p-aminobenzoic acid, 2.5 g yeast extract and 60 g glucose. The flask was incubated at 36 °C in a shaker with gentle agitation.

4.2.2 ABE fermentations

Two chemicals were tested as extractive agents in biphasic fermentations: a vegetable oil (pomace olive oil, PO) and a C12 based Guerbet alcohol (2-butyl-1-octanol, 2B1O). Batch and fed-batch fermentations were carried out in a 2-L stirred tank reactor BIOSTAT B plus (Sartorius Stedim Biotech SA). The vessel was filled with a total volume of 1.6 L. The liquid was inoculated with 100 mL of cells growing at maximal growth rate. The amount of solvent was fixed taking into account the expected partition efficiency for butanol reported in Chapter 2. Thus, the ratio organic: aqueous phase for biphasic fermentations with PO and 2B1O were 1:1 (v/v) and 0.25:1 (v/v), respectively. The system was previously purged with N_2. All fermentations were carried out at low stirring rate (40 rpm), maintaining a clear separation between phases. Each phase was provided with a mechanical stirrer sharing a common rotation axis. The reactor vessel was adapted with a sampling device for both aqueous and organic phase. The temperature was fixed at 35 ºC. The system was then autoclaved at 121 °C during 20 minutes before inoculation. In fed-batch operation, pulses of a concentrated feed of 300 g glucose/L with yeast extract and ammonium acetate scaled to the same proportion were applied. pH

was set to 6 at the beginning of the fermentation, and then it varied freely. For all tests initial glucose was fixed at 90 g/L.

4.2.3 Flow cytometry

Samples were harvested from culture medium and separated from supernatant by centrifugation at 2800x g during 10 min, washed twice with distilled water, suspended in PBS solution and stored at 4ºC. Before staining, samples were filtered through a 30 µm pore size membrane and diluted to $5x10^6$cells m/L in PBS. Then, 100 µL of PI solution (0.02 $g \cdot L^{-1}$in PBS) were added to 1 mL of sample and dark-incubated for 5 minutes at 4 °C. Control and stained samples were analyzed in a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Inc. Brea, CA, USA) equipped with an Argon laser at 488 nm at the CACTI facilities of the University of Vigo (Vigo, Spain). Forward and side scattering, and red fluorescence (FL3, 620 nm BP filter) signals were recorded using logarithmic amplification, and the data was stored as list mode data files (LMD) for subsequent analysis. LMD files store data according to the FCS 2.0 standard (standard data file for flow cytometry, version 2.0), as described by the Society for Analytical Cytology (1990). Flow rate was set at medium speed and 30,000 cells were analyzed per sample. WEASEL flow cytometry analysis software v3.0.2 (Walter and Elisa Hall Institute, Melbourne, Australia) was used to determine the fraction of each cell type and to analyze cell membrane permeability.

4.2.4 Other analytical protocols

Aqueous and organic phase compositions were determined by gas chromatography using HP6890 GC, with N_2 as carrier gas and a flame ionization detector. The GC was equipped with an Agilent DAB-WAX column (Ref. Nº 122-7032) of 30 m length and 0.25 mm diameter. The oven was kept at 70 °C for 3 min and, thereafter, temperature was increased up to 200 °C at a gradient of 50 °C/min. A different calibration curve for each compound in aqueous phase (solvents, acids) was generated. In the case of organic samples, hexane was combined at a ratio of 1:1 (v/v) to the organic aliquot being analyzed. The objective was to ensure a single homogeneous sample at room temperature before GC analysis. Quantification of glucose was performed using an enzymatic kit (Glucose-TR GOD-POD, Ref.1001190, Spinreact, GI, Spain).

A JEOL JEM-1011 (JEOL Ltd., Akishima Japan) transmission electron microscope (TEM) was used to evaluate morphological differences between different cell types. For that purpose, cells were taken from the same samples stored for flow cytometric analyses, fixed with glutaraldehyde, embedded, and sectioned using an ultramicrotome.

The typical standard deviation of the analytical protocols used for measuring the percentage of each type of cells (flow cytometry) is 0.23, for the concentration of substrate (glucose enzymatic kit) is 0.04 and for products (acids and solvents, using GC-FID) is 0.26.

In this study, the total concentration of suspended biomass was determined in terms of dry cell weight (DCW) by lyophilization (freeze-drying) of the sample with a LABONCO Freezone 1 apparatus. Optical density was determined in a Shimadzu UV-1800 spectrophotometer at 600 nm relative to distilled water. A calibration curve relating OD_{600} and bacterial cell concentration DCW (g/L) gave 0.477 g of DCW per liter for an OD of 1.0.

4.2.5 Statistical analysis

The time at which 50% of the initial substrate was consumed (t50), the concentration of total solvents at t50 and the area under the glucose concentration curve (AUC) were calculated for the three fermentations. First, for each fermentation, experimental glucose concentration was fitted to a three-parameter logistic equation using the software program fityk version 0.8.6 (http://fityk.nieto.pl/). In all cases, the regression coefficients (r^2) were higher than 0.95. Then, the obtained equation was used to calculate t50 and the total solvent concentration at this time was obtained by interpolation of the experimental data. Mean values and standard deviations of the results obtained from the three fermentations were subsequently calculated. Finally, the AUC, its standard deviation and confidence interval at 95% level were calculated using the package PK from the statistical software program R version 2.8.1 (The R Foundation for Statistical Computing).

4.3 Results and discussion

4.3.1 Product profiles of control and biphasic ABE fermentations

Control (solvent free) ABE fermentation

In conventional ABE fermentation, solvent production and *Clostridia* metabolic activity cease when critical threshold concentration of inhibitory metabolites (mainly butanol) is attained. In this study, during control batch ABE fermentation, butyric acid was generated during the acidogenic phase and its concentration increased up to 2-2.5· before being reconverted into butanol. Culture metabolic switch was well determined by the first inversion of pH trend (data not shown) at 15h of batch test. Solvents (butanol, acetone and ethanol) were produced from the beginning of solventogenic phase simultaneously with substrate depletion and up to around 40 h, when aqueous butanol concentration exceeds 12 g/L (Figure 4.2A).

A total of 21.6 g solvents/L was obtained, and up to 70% of initial substrate was consumed.

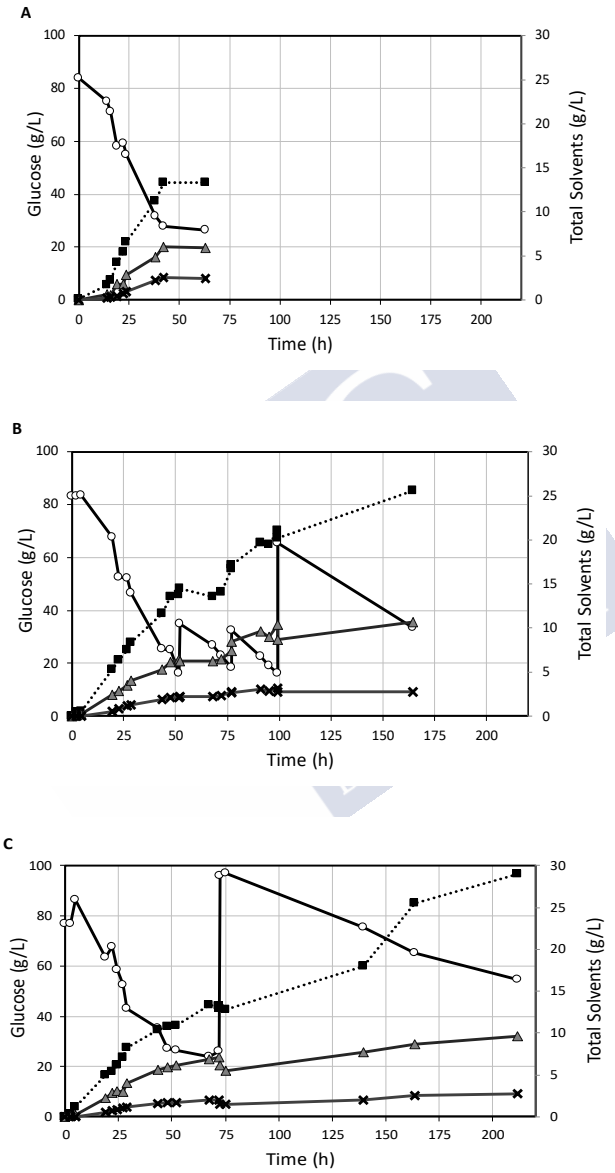


Figure 4.2 Concentration profiles of glucose (void circle) and total solvents (butanol (black square), acetone (grey triangle) and ethanol (diagonal cross)) during batch conventional (A) and extractive ABE fermentations with Pomace Olive Oil (B) and 2B1O (C).

Pomace Olive Oil Fed-batch extractive fermentation

In this fermentation (Figure 4.2B), three pulses of glucose were added to the reactor at 50 h, 77 h and 100 h. Glucose uptake rate was maintained after 48 h due to end-product inhibition alleviation by partial butanol removal into the organic phase. Before the first addition pulse, a total of 80% of initial glucose was consumed (which is 10% higher than control fermentation at the same time thanks to end-product inhibition alleviation). At the first and the third feed pulses, 200 mL of organic phase were replaced by fresh vegetable oil (partial regeneration of the extractant phase), which resulted in an extension of the production phase period. Consequently, production of solvents was maintained up to the end of the fermentation and total butanol production was increased by 69%, with respect to the control. After the third fed-pulse (100 h) productivity and glucose uptake rate decreased. Indeed, at that time, aqueous butanol concentration attained the typical threshold concentration in Clostridia (10 g/L). Butanol continuous to be produced up to the end of the fermentation (Figure 4.2B) but at a lower rate.

2-Butyl-1-Octanol Fed-batch extractive fermentation

In this case (Figure 4.2C), only a fed-batch pulse of concentrated glucose was applied at 75 h. At that moment, the organic phase was partially regenerated by replacing 200 mL of fresh 2B1O. Initial glucose uptake rate was similar to that of the control fermentation and pomace-oil extractive fermentation according to statistical analysis. The standard deviations of AUC, t50 and the concentration of total solvents at t50 were 0.43, 5.54 and 6.92%, respectively. Considering these parameters as indicators of reproducibility between fermentations, the difference between the first period of ABE fermentation profiles were not significant. Nevertheless, glucose consumption rate was significantly reduced after the pulse addition. It was expected that glucose consumption could go beyond 70% of its initial value (as per the control test), because removal of inhibitory product should enhance metabolic activity. However, glucose consumption in extractive fermentation also stabilized after around 40 h. The production of solvents was maintained through the entire fermentation, and more than 40 g solvent/L were produced. Compared to control fermentation, final overproduction of butanol was around 72% in biphasic fermentation with 2B1O (Figure 4.2C). Nevertheless, productivity decreased after the fed-batch pulse (at 75 h), which could not be explained in terms of high or critical aqueous butanol concentration since it was kept under 4 g/L all over the fermentation. Butanol production could be affected by dissolved 2B1O in aqueous phase (log P < 4 for this solvent) and/or by biphasic toxicity.

In fact, 2B1O extractive fermentation in stirred 2L bioreactor presented degraded performance compared to previous work carried out in sealed-bottles

(Chapter 3). In the latter, glucose was depleted, and maximal butanol productivity was enhanced over that of the control fermentation, after a marked culture adaptation lag time. In the bioreactor, mass transfer might have been enhanced by increasing the L/D ratio and agitation. However, in both experiences (flask and bioreactor), liquid-liquid equilibria were immediately attained through the fermentation, thus no mass transfer limitations occurred. This implies that dissolved toxicity effect is similar in both scale experiences, and it can be assumed that dissolved 2B1O molecules in sealed flasks attain cells as quick as in the bioreactor. On the contrary, biphasic toxicity effect might be enhanced in stirred reactor (more exchange surface between phases) and would explain degraded productivity. However, the lag time observed in the flask fermentations did not occur at bioreactor scale.

Table 4.1 compares the performance of the control and extractive fermentations at bioreactor. Butanol productivity values were estimated in batch control considering lag time and up to the final value. In fed-batch extractive fermentation, time related parameters were calculated before the first fed-glucose pulse for comparison purposes. Frequency of glucose fed-pulses in the case of PO extractive fermentation was not optimized, which could lead to progressive accumulation of acid in the bioreactor (Parnas et al. 2011). In both extractive fermentations, time related parameters (glucose uptake rate and productivities) were degraded in the second half of the fermentation. Butanol yield was improved by 35% in the extractive fermentation with 2B1O over solvent-free control, in agreement with previous work.

Table 4.1. Summary of results in 2L stirred bioreactor

	Test Control	*Test VO*	*Test 2B1O*
Mode	Batch	Fed Batch	Fed Batch
Glucose consumption, g/L	57.41	131.93	92.39
Butanol, g/L	13.30	25.57	29.02
Acetone, g/L	5.89	10.68	9.61
Ethanol, g/L	2.46	2.80	2.75
ABE, g/L	21.65	39.05	41.38
Butanol yield, g/g	0.23	0.19	0.31
ABE yield g/g	0.38	0.30	0.45
*Butanol productivity g/l/h**	0.27	0.28	0.21

4.3.2 Biomass growth in control and extractive fermentations

Biomass changes in the bioreactor, measured by dry cell weight (DCW) and optical density (OD), are depicted in Figure 4.3 for the control and extractive fermentations. It is important to note that only suspended biomass, and not total biomass, was quantified. In the control fermentation, cell growth was observed up to the metabolic switch (before 20 h), when it started to diminish sharply (56% decrease in 10 h). This decrease in OD just after the exponential growth phase has already been observed with *Clostridium acetobutylicum* ATCC 824 (Monot et al. 1984, Liu et al. 2015), and it has been related to a spontaneous large autolysis of sporulating cells. Liu et al. (2015) determined that autolysis benefits sporulation, since the lysed material furnishes nutrients to the intact sporulating cells, thus guaranteeing survival.

After maximal cell growth stopped, the lysed material aggregation around the baffles, impellers and the agitation axis, and covering partially the internal bioreactor walls, was clearly appreciated in the control and extractive fermentations.

The suspended biomass concentration of PO extractive fermentation was lower during the first round of acidogenesis-solventogenesis (< 50 h), and OD decreased just before the first glucose pulse, probably due to the cell lysis. After the pulse, biomass growth was enhanced. At around 70 h, it started to decrease again, but this tendency was inverted after the addition of the second pulse at 77 h. Biomass growth was maintained up to 90 h, when cell concentration sharply decreased. After the last pulse (100 h) no increase in suspended cell concentration was appreciated. High growth rates corresponded to both acids and solvents production periods (Figure 4.2).

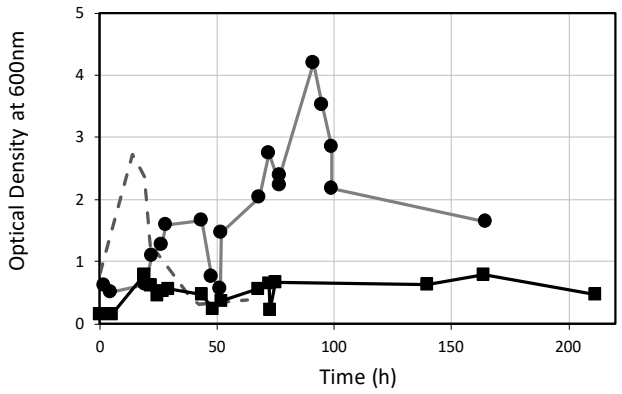


Figure 4.3. Evolution of the optical density during batch conventional (dashed line) and extractive ABE fermentations with Pomace Olive Oil (black circle) and 2B1O (black square).

OD of the 2B1O extractive fermentation was lower than that of the control and PO extractive fermentations. A higher tendency of cells to aggregate was observed in the reactor, similarly to that observed in the flask experiments with the same solvent (Chapter 3). This could partially explain the lower suspended cell concentration. Another explanation is that higher solvent yields have been reported in cultures challenged by butanol addition (Kanchanatawee et al. 1991, Wang et al. 2016). After analyzing cell viability and biomass profiles of batch fermentations it was concluded that under butanol stress a lower number of cells produced the same amount of butanol (Branska et al. 2018).

In the case of 2B1O extractive fermentation, aqueous butanol was not responsible of the limited microbial growth, since it remained under the inhibitory threshold. However, it has been previously stated that linear alcohol share deleterious effect on microorganism (Ingram and Butke 1984), and dissolved molecules of 2B1O might cause the same effect as that of butanol on Clostridia, as discussed in Chapter 3. Biomass growth stopped after the fed pulse at 75 h, even when solvent production was maintained up to the end of the fermentation.

4.3.3 Determination of different morphological cell types of *C. acetobutylicum*

The classification of the different cell types as function of forward and side scattering is presented in Figure 4.4. The assignation of each FSC-SSC region in the dot-plot diagram to a specific type of cell took into account the work of Tracy et al. (2008), and also microscopy observations conducted on the samples that were subsequently analyzed by FC (Figure 4.5).

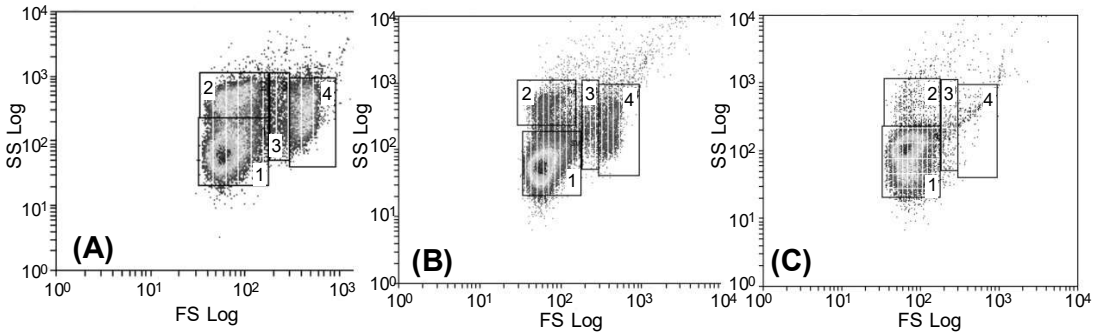


Figure 4.4. Different C. acetobutylicum cell types as function of forward and side scattering measured by flow cytometry. (1) early stationary vegetative cells, (2) late stationary vegetative cells and clostridia, (3) mostly clostridial cells + forespores, and (4) endospore + free spores. Cell types were identified in the following samples: (A) heated shocked spores, (B) pre-culture medium after 50 h of culture and (C) culture medium after 20 h of culture (high growing cells). TEM photographs of the cell types observed during the production of butanol by C. acetobutylicum. Numbers assigned to every cell type are the same as those used in Figure 4.5

Four different regions, corresponding to different cell predominant phenotypes were identified (same numbering as in Figures 4.4 and 4.5): (1) early vegetative rod-shaped cells, (2) late vegetative rod-shaped cells and some clostridial forms presenting higher SS which reflects internal granulose accumulation, (3) mostly clostridial cells + forespore grouped in a dispersed region with higher FS intensity, and (4) sporulating forms: endospore + free spore cells with the highest FS. Quantification of the percentage of each cell type, corresponding to the four regions was carried out considering this classification.

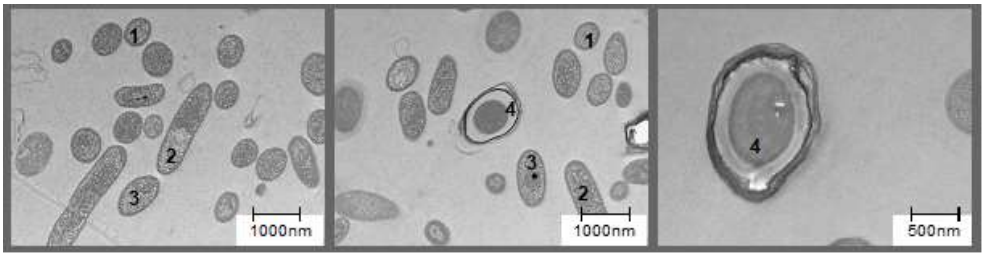


Figure 4.5 TEM photographs of the cell types observed during the production of butanol by C. acetobutylicum. Numbers assigned to every cell type are the same as those used in Figure 4.4

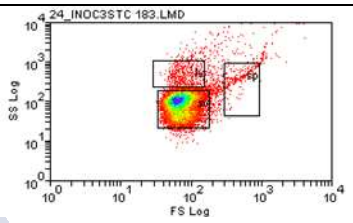
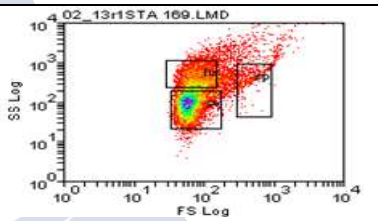
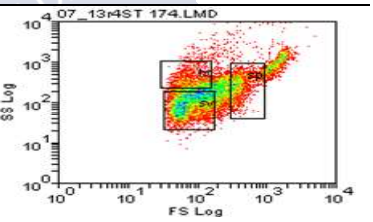
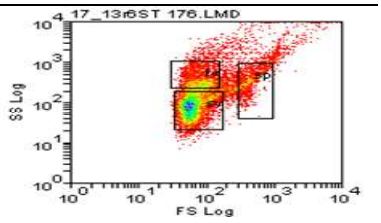
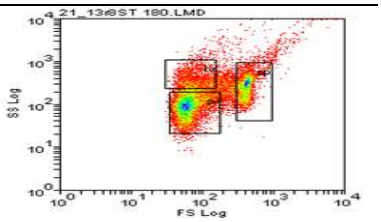
Population dynamics of C. acetobutylicum during conventional ABE fermentation

The quantitative evolution of cell types of *C. acetobutylicum* determined by FC is presented in Table 4.2, where FC plots corresponding to different characteristic times of conventional batch fermentation are included. Cell types were represented according to the classification shown in Figure 4.4. Cells belonging to region 3 (mostly clostridial + forespore cells) were a minor group and were not included in this analysis.

It is interesting to note that the sample at initial time (t= 0h) depicted a different subpopulation distribution than the inoculum. While the latest (high growing cells) contained almost exclusively soon vegetative cells (> 96%), the first sample (0 h) revealed a higher fraction of sporulating cells (8%), indicating possibly culture evolution during transfer and inoculation procedures. During acidogenic phase (up to 10 h), the percentage of total vegetative cells (SV + LV) increased to 95% at the metabolic switch (Table 4.2) At the same time, the percentage of sporulating cells decreased, probably due to germination.

At 22 h, just after the metabolic switch and during the high solvent rate production, an unexpected increase of the fraction of sporulating cells was observed. Nevertheless, a closer look at the FSC/SSC plot corresponding to this point shows a high dispersion zone, which may have impacted the correct assignation of the events in region 4 as sporulating cells. Tracy et al. (2008) observed the same phenomena and attributed this high FSC/SSC dispersed zone to the bulk of inactive lysed cells that were more propitious to form aggregates. This assumption is supported by the biomass evolution data discussed above (OD600, Figure 4.3). In fact, a sharp OD decrease was initiated concomitantly. This has been associated with a quick and spontaneous autolysis in batch fermentation with *C. acetobutylicum* ATCC 824 (Liu et al. 2015) and could explain the immediate presence of lysed material in the broth. Liu et al. (2015) determined that the autolysis process is selective, affecting only inactive cells and benefits further on-going sporulation.

Table 4.2. Microbial dynamics during conventional batch fermentation

Culture time	*%Soon Vegetaives (1)*	*%Late Vegetatives (2)*	*%Sporulating (4)*	*FSC/SSC dot-plot*
Inoculum	96.3	2.7	1.3	
14 h (Metabolic Switch)	71.9	22.1	2.8	
22h (high solvent rate)	63.4	14.6	15.9	
38h (Butanol at inhibition threshold)	70.4	17.8	7.4	
63h (End of the culture)	57.2	12.5	23.8	

After 40 h of batch control fermentation (Figure 4.6), the fraction of sporulating cells started to increase and at 63 h it reached up to more than 20%. This coincided with the decrease of the percentage of vegetative cells (from > 95% at 20 h to 70% at the end of the fermentation) and the cease of metabolic activity due to the presence of inhibitory concentrations of total solvents. It is interesting to note that the evolution of microbial subpopulations continued even after the metabolic activity ceased at about 40 h (Figure 4.2). Thus, the highest increase of sporulating cells happened between 40 and 60 h, probably in detriment of non-active vegetative cells.

Tracy et al. (2008) compared the evolution of cell populations determined by FC with butanol flux and concluded that the latter correlated to the fraction of vegetative-rod shaped cells. In Figure 4.6, the evolution of butanol flux is depicted together with that of the percentage of each subpopulation. Butanol flux was determined as the change of molar butanol concentration over time (mM/ h)). It is observed that the fraction of vegetative cells and the butanol flux evolved in synchrony through the fermentation. This corroborates that a vegetative cell phenotype is responsible for butanol production in ABE fermentation.

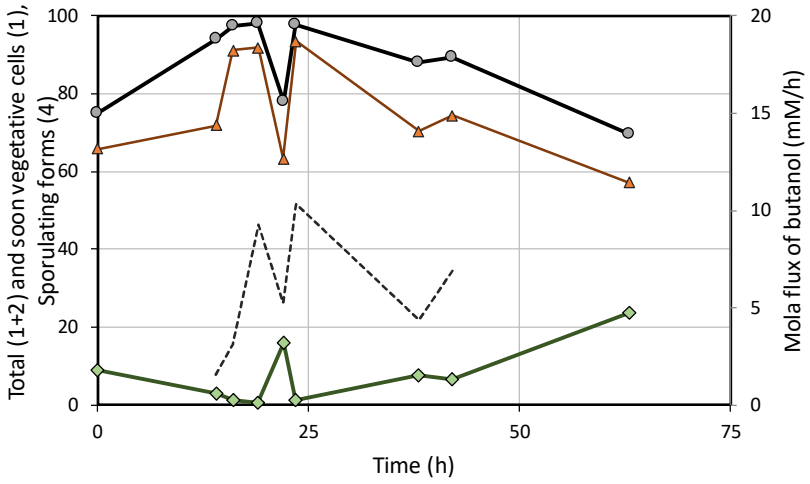


Figure 4.6. Evolution of the percentage of total vegetative (grey circle), early vegetative (orange triangle)) and sporulating (green diamond) cells during batch conventional. Variation of molar flux of butanol (discontinuous grey line)

Population dynamics of C. acetobutylicum during PO extractive fermentation

During fed-batch PO extractive fermentation (Figure 4.7), a peak in the fraction of sporulating forms (39% at 52 h) was observed just before the first fed-batch pulse, which was coincident with a sharp OD decrease (Figure 4.3). However, opposite to that occurred in the control fermentation, metabolic activity did not cease. At this point, aqueous butanol concentration was not at the inhibitory threshold (~6 g/L). Afterwards and until the end of the fermentation, sporulating forms remained at around 10%, while the presence of vegetative cells was higher than 85%. These results demonstrate that during extractive ABE fermentations it is possible to maintain a continuous butanol production by cultures composed mainly of vegetative cells, in agreement with Tracy et al. (2008) that have previously proposed that a clostridial-form cell precursor (vegetative cell) is the major solvent producer. To the best of our knowledge, the flow cytometry results shown in the present study are the first demonstrating that butanol production by *C. acetobutylicum* during extractive fermentations is also carried out mainly by vegetative cells.

At the end of the fermentation (from 100 h), aqueous butanol exceeded inhibitory threshold (12 g/L) and metabolic activity ceased. Nevertheless, sporulating forms did not increase as expected. Liu et al. (2015) determined the importance of lysed cellular material as nutrients for sporulating population to be further developed. In this study, a viscous extracellular compound issued from cell lysis was observed in the vegetable oil phase. Thus, the transfer of this lysed cellular material from the aqueous to the organic phase would have limited the sporulating cells development at the end of the fermentation.

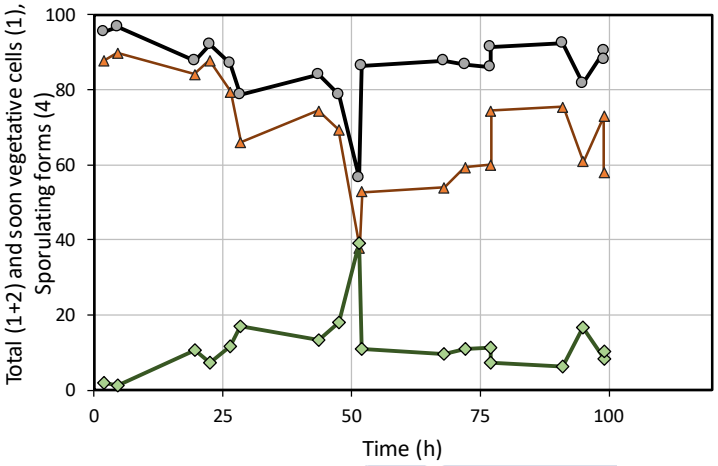


Figure 4.7. Evolution of the percentage of total vegetative (grey circle), early vegetative (orange triangle)) and sporulating (green diamond) cells fed-batch extractive fermentation with POO

Population dynamics of C. acetobutylicum during 2B1O extractive fermentation

The evolution of cell types during 2B1O extractive fermentation is depicted in Figure 4.8. From 30 h onwards, the percentage of sporulating forms began to increase from 10 to 40% at the end of the fermentation, while at the same time the vegetative cells decreased from 85 to 50%. In the case of the control fermentation this change was caused by the presence of solvents at inhibitory concentrations. However, during 2B1O fermentation, the inhibitory butanol threshold concentration in the aqueous phase was never reached, being its maximal value 3.9 g/L. A possible explanation of this behavior is that *C. acetobutylicum* could had been affected in the reactor by dissolved 2B1O in aqueous phase or by biphasic toxicity, demonstrating that 2B1O presents lower biocompatibility towards *C. acetobutylicum* than PO. In a previous work, Gonzalez-Peñas et al. (2014), demonstrated that both 2B1O and PO are biocompatible when assayed at small scale experiments carried out without mixing. In sealed-bottle flask fermentations (Chapter 3) lag time occurred, but cells finally adapted, and glucose was depleted. The more favorable geometry for liquid-liquid transfer of the bioreactor vessel (lower height: diameter ratio) and the mechanical stirring applied in this study may have affected negatively the biocompatibility of 2B1O. Offeman et al. (2008) assayed the toxicity of beta-branched alcohols in fermentations with a commercial strain of *S. cerevisiae* and found that all C8-C12 alcohols except 2B1O were toxic to the cells. However, intermediate values of ethanol production and glucose

consumption between those found for toxic and non-toxic solvents, were obtained with 2B1O.

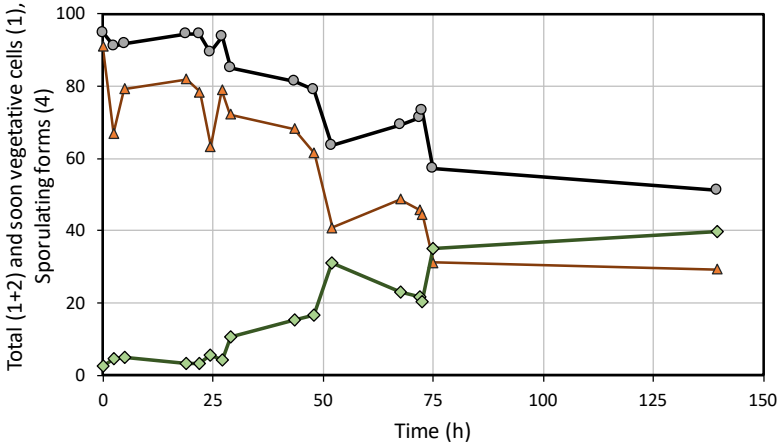


Figure 4.8. Evolution of the percentage of total vegetative (grey circle), early vegetative (orange triangle)) and sporulating (green diamond) cells fed-batch extractive fermentation with 2B1O

4.3.3 Cell permeability.

Figure 4.9 presents the evolution of cells that are permeable to PI. Permeability of PI has been used as an indicator of cell viability in many FC studies with different microorganisms. Cells that do not permit PI to pass the membrane cell (PI- cells) appeared in the region of the control cells that were not stained, while the cells with modified membrane permeability (PI+ cells) presented a more intense fluorescence. During the first 48 h, the highest percentage of PI+ cells was observed in the conventional fermentation, while the lowest one was attained with PO. In the case of both extractive fermentations, a significant presence of PI+ cells was observed after 48 h, reaching values higher than those observed in the control fermentation. In the control fermentation, the metabolic activity stopped at around 40 h, however the percentage of membrane permeable cells did not increase after this time. On the contrary, during the PO extractive fermentation, a significant and continuous increase of the percentage of permeable cells was observed from 48 h on, coincident with a period of glucose consumption and production of solvents. This would indicate that even though cells were permeable to PI, they maintained a high metabolic activity. According to these data, PI permeability does not correlate with butanol productivity, but with the evolution of different phenotypes. Analyzing together all of these results it was concluded that it is not possible to

correlate cell viability of *C. acetobutylicum* to the fraction of permeable cells stained with PI by using flow cytometry. In accordance to these results, Jones et al. (2008) followed *Clostridium* fermentation time course by phase-contrast microscopy coupled to double PI and Syto-9 staining and concluded that staining changes respond to membrane modifications induced by medium conditions (acids, solvents) and do not reflect cell viability.

PI, combined with Syto-9, has been successfully used as a supporting tool to identify and quantify the different morphological states of *Clostridium acetobutylicum* ATCC 824 (Tracy et al. 2008). However, the use of permeability probes in Clostridia cultures is controversial because there have been other studies in which different staining probes were successfully applied to monitor and quantify viability changes in cultures of *C. beijerinckii* (Branska et al. 2018) or *C. pasteraunium* (Linhová et al. 2010).

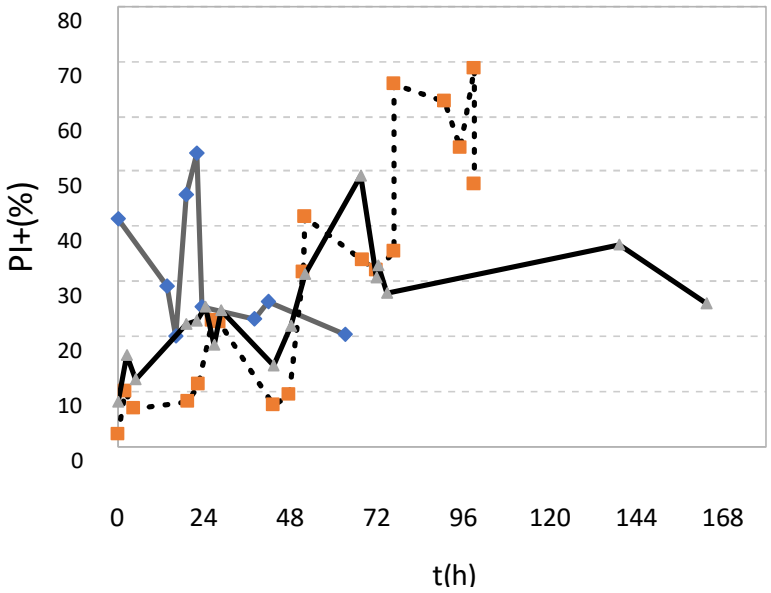


Figure 4.9. Evolution of the percentage of permeable cells (PI+) determined by using FC during control batch (blue diamond) and extractive fermentations with PO (orange square) and 2B1O (grey triangle).

4.4 Conclusions

Flow cytometry is a useful technique for measuring the morphological changes of *Clostridia* culture occurring during ABE liquid-liquid extractive fermentations

with solvents presenting different biocompatibilities. Besides, it was found that in fed-batch ABE extractive fermentation with pomace olive oil, a continuous butanol production can be achieved by predominantly vegetative cells and low sporulating forms participation.

Although changes in membrane cell permeability due to phenotype evolution were observed by PI staining, a correlation between this parameter and butanol productivity or cell viability could not be found.

Research on the application of different staining probes, together with flow cytometry complementary cell-sorting techniques would allow to further investigate the physiological heterogeneity in ABE extractive fermentation with solvents of different nature.

Some useful information about clostridia culture heterogeneity evolution during ABE fermentation that was acquired in this chapter will set the basis for further modeling of the process.

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5. Modeling batch ABE fermentation with microbial population dynamics

Transient product spectrum during the batch Acetone-Butanol-Ethanol fermentation evolves in response to the pH-dependent *Clostridium acetobutylicum* metabolic variations. Previous works evidenced the coexistence of distinct subpopulations of the microbial culture in the bioreactor, each one with specific metabolic activity. However, the culture heterogeneity has longtime been ignored in ABE mathematical modeling. Here, a dynamic model for batch ABE fermentation kinetics is developed under the hypothesis of an existing specific solventogenic phenotype, based on a simplified ABE metabolic network. Particularly, the metabolic switches in simulated batch operation are described as triggered by the partial conversion of acid vegetative cells into a solventogenic and sporulating subpopulation. The proposed model adequately describes the culture inhibition and the dynamic behavior of all the extracellular metabolites in a batch culture with both free and controlled pH. Subsequently, *in situ* concomitant liquid-extraction was integrated in the model. The model simulations however do not predict well when applied to extractive fermentation using solvents. This indicates that phenomena are at play beyond the simple transport of products between aqueous and organic phases. Presumably complex solvent toxicity effects on the Clostridia cell cycle may be relevant and must be accounted for in further modeling developments

Keywords: *Clostridium acetobutylicum,* batch culture, metabolic switch, microbial heterogeneity, model

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OUTLINE

5.1 Introduction

5.1.1 Modeling of a fermentation process

Modeling is a multipurpose useful tool for any process. On one hand, theoretical model aims at translating physical and chemical phenomena into mathematical equations, and this helps to more deeply understand the process, which is the basis for any further intensification and optimization. Sometimes the knowledge of the process is limited, data are not easily available, or theoretical degree of complexity might be difficult to hand. In these cases, assumptions are made, and simplified models can be established. But even empirical models help to identify the influence of each variable on the overall process performance and their inter-dependency. In every case, modeling allows to screen operating conditions, reducing experimental investigations (which are often not only time consuming but involve significant cost). Model-based simulation study may allow to find best flow conditions (operation mode) for a process, and to optimize reactor design. Furthermore, a model can also help in the process scale up, from laboratory to industrial size reactor, which is a quite complex task. Indeed, extrapolation implies to predict the response of the variable of the process to conditions not necessarily explored at small scale. This is more easily managed if a reliable model is available.

The usefulness of modeling is particularly true for fermentation processes, where physical phenomena (transport and diffusion of nutriments and metabolites, shear rate forces induced by mechanical agitation, heat balances in the bioreactor, etc.) must be coupled with intrinsic complex biochemical pathways, specific for each microorganism. This means not only to create a more complete system of equations but also to consider mutual interactions. In other words, prior to model establishment, it might be important to quantify the reciprocal influence of physical and chemical variables of the process (pH, T, agitation rate…) on its biology (metabolism and physiologic state of the biocatalyst).

A classical structure of a mathematical model for a biochemical process comprises three parts: 1) the description of the metabolic pathway of the microorganism involved, 2) the development of the rate equations for the biomass production and the uptake/formation of substrate and products respectively, and 3) mass balance of the reactional volume for each metabolite.

Microbial growth modeling in bioreactors have been classified according to their complexity type and degree (Nielsen, 2006). Unsegregated models are most common and consider the cells as a unique population with an average cell description. Within this category, unstructured models, which consider the cell as a “black box”, and thus do not look inside it, are opposed to structured models, that consider full stoichiometry, including intracellular kinetics. On the other hand,

segregated models recognize differences in a pure culture population, considering that not all the cells in the bioreactor are identical; in these models, at least one distribution characteristic of the microbial cells must appear (from size to more complex cell population balances). The chosen complexity degree depends on the aim of the metabolic exercise.

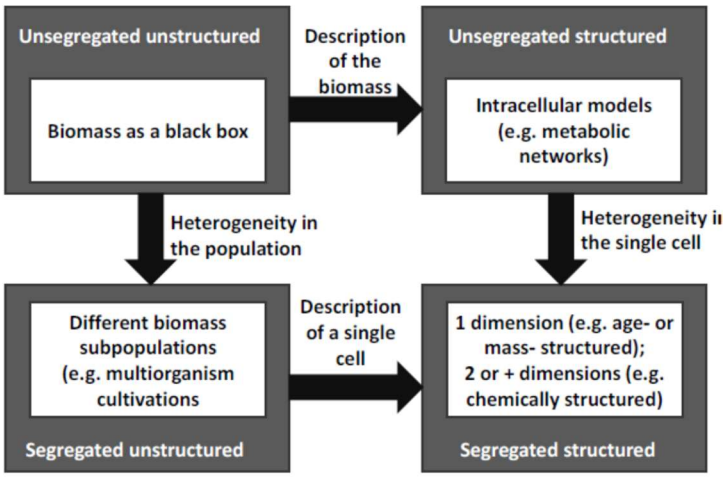

Figure 5.1. Classification of microbial mathematical model from (Gernaey, 2015)

5.1.2 The complex cell cycle of the ABE fermentation

As stated in previous sections, (Chapter 1, Chapter 4), Acetone-Butanol-Ethanol fermentation by Clostridia microorganisms is a complex process that involves at least two phases: acidogenic (growth associated production of carboxylic acids with a consequent decrease of broth pH) and solventogenic (assimilation of acids into solvents, during a pseudo stationary phase) (Lütke-Everlosh & Bahl, 2011). The enzymes involved in this second part of the metabolism are triggered by several factors (external pH, intracellular undissociated acids), the metabolic switch has also been considered as an adaptive response of the cells to the low pH (Jones & Woods, 1986). In parallel to these metabolic changes, the microorganism follows a quite complex cell cycle. During acid production (exponential microbial growth), the so-called vegetative cells are predominant. They are highly motile and rod shaped. At the onset of solventogenesis, some of these cells evolve into fattened cigar-shaped clostridial cells which are precursor of sporulating phenotypes (forespore/endospore). The free spores are finally ejected into the broth and have no metabolic activity but could re germinate if conditions are adequate (Schuster, et al., 1998).

Therefore, the clostridia culture during ABE fermentation is intrinsically heterogenous. The coexistence of distinct subpopulations of the microbial culture in the bioreactor during ABE fermentation, each one with specific phenotype and metabolic activity associated, has been repeatedly emerged from experimental evidence in literature. Especially in suspension cells in continuous flow, where butanol concentration is kept well under toxicity threshold, heterogenous population led to observed oscillations in the levels of acids and solvents (Maddox et al 89, Gapes et al 2000, Parnas et al 2011).

In standard batch fermentations, where high butanol production rate led to toxic concentration concomitantly to glucose depletion, there is a classical temporal association between solventogenesis, stationary phase and sporulation, and consequently, non-growing "clostridia" phenotype has been longtime considered as the solvent production responsible in literature (Schuster et al 1998, Jones and Woods, 1986). Nevertheless, it has been reported that all these processes (solvent production, sporulation) share regulatory mechanism but are not necessarily linked (Millat et al 2017), challenging this long-accepted view. Indeed, solvent formation in batch fermentation batch culture has been observed before the transition to stationary phase (Monot et al 1984). Summarizing, the role of different cell types co-existing in the culture has been longtime discussed although a simple connection between cell types and fermentation product has not been well established.

Tracy et al. (2008) used Flow cytometry (FC) and fluorescence-assisted cell-sorting (FACS) techniques to investigate clostridia heterogeneity in batch fermentation. By correlating the cell-morphology evolution with the metabolite concentration profiles in the broth, these authors stated that a vegetative cell phenotype – precursor to clostridia-form cell- was responsible of butanol production in ABE fermentation broth with *Clostridium acetobutylicum*. In Chapter 4 of this thesis, FC was also applied to monitor population dynamics during ABE extractive fermentation, and it was demonstrated that butanol production could be achieved through fed-batch fermentation by mainly only vegetative cells.

Under the hypothesis of an existing growing solventogenic phenotype, modeling the metabolism in ABE fermentations might be subject to different constraint that those applied in most of existing models. This new approach was somehow considered in the model developed by Millat et al 2013. This model revealed that the pH-induced metabolic shift in continuous culture with *Clostridium acetobutylicum* was governed by the switch of two growing distinct pH-dependent subpopulations (acidogenic and solventogenic respectively).

5.1.3 ABE extractive fermentation

Another important issue of ABE fermentation is that it suffers from strong product inhibition during both phases of the fermentation. Therefore, butyric acid inhibits microbial growth during acidogenesis, and solvent production ceases when butanol (main inhibitory product) reaches a threshold critical concentration (around 10-12 g/L for *Clostridium acetobutylicum* ATCC824). It is reported that fluidity of the cell membrane increases in the presence of butanol, leading to a destabilization of the cell membrane function and stopping finally metabolic activity (Jones & Woods, 1986).

In order to overcome this important limitation, the application of an In Situ Product Removal (ISPR) system has been encouraged. In that system, an external agent is added to the bioreactor with the aim to remove the inhibitory butanol as soon as it is produced, alleviating the inhibition and boosting overall performance. Several techniques have been investigated for the last decades: gas stripping, perstraction, pervaporation, liquid-liquid extraction… (Outram, et al., 2017). In the latest, a water insoluble extracting compound, with ideally high alcohol capacity and selectivity, is added to the fermentation broth. Both phases are then easily separated. However, the optimal solvent is a compromise between alcohol distribution coefficient and biocompatibility towards the microorganism (Daugulis et al. 1991). In fact, apart from toxicity issues, the solvent phase in the bioreactor might have non-negligible influence on ABE fermentation metabolism (Chapter 3). Moreover, not only final products but also intermediates acids are concomitantly extracted into the extractant, modifying then thermodynamics of the system. It has been reported that final yield structure can be modified with solvents presenting higher partition coefficient for butyric and butanol. (Chapter 2, Chapter 3).

5.1.4 Background on dynamic ABE models

Following the classification presented in Figure 5.1, some of existing models of ABE fermentation are compiled in Table 5.1, where main features of each reference are pointed out. ABE fermentation with immobilized cells models are not considered here, since the structure of these models is more complex and intrinsically different. Indeed, it has been stated that the quorum sensing phenomena lead to specific microbial behavior, in addition to multidimensionality, diffusion and transport, adsorption/attachment/detachment dynamic, etc. (Horn & Lackner, 2014). This broad complexity deserves a separated review.

In most of the published ABE models, the rate expression for biomass growth is based on Monod kinetic model, the specific growth rate, including a substrate saturation factor. ABE fermentation is clearly gouverned by end-product inhibition, thus, a term accounting for this phenomena is systematically included in the

kinetical model. Empirical modeling of end-product inhibition phenomena has been done in literature using linear, exponential, parabolic law kinetics under fermentation conditions. (Mulchandani & Luong, 1989) published a complete review of different unstructured and empirical kinetic models representing general microbial substrate and product inhibition (not specifically for ABE production). Yang & Tsao (1994) developed an empirical mathematical expression for inhibition function on biomass growth rate in ABE fermentation, based on a fractional factorial design, accounting for pH, butanol and butyric acid influence on growth inhibition.

The flow operating mode of the process will be reflected in the mass balances developed for each metabolite. It has been pointed out (Napoli, et al., 2011) that simultaneous production of acids and solvents are more likely to occur in continuous mode operation, with at least acidogenic and solventogenic cultures coexisting in the bioreactor. This make the continuous ABE production a more complicated system to be modeled and investigated, if no biomass distinction types are considered. However, as discussed above, it has been proved that coexisting different microbial subpopulation also occur in batch mode, even if classical subsequent biphasic behavior makes it more difficult to appreciate.

As seen before, the metabolic network of ABE fermentation involves multiple interdependent reactions, with complex kinetics and many intracellular intermediates. In some of the reported works, several reactions are lumped together to reduce the complexity level of the model. Indeed, benefits in bioreactor design, scale up and process optimization may also be derived from simpler unstructured models (Napoli et al. 2011). In many cases, only the rate equations for main extracellular metabolites, biomass and substrate are written. Some of the first reported models on ABE fermentation are those developed by Volesky and collaborators (Votruba et al. 1985, Yerushalmi et al. 1986). Based on simplified metabolic pathway, these models predicted the main metabolites evolution in batch process and captured well the biphasic behavior of ABE fermentation, by the consideration of a physiological maker of the culture. Nevertheless, these first models did not consider the effect of the pH in the metabolic network. Srivastava and Volesky (1990) improved the existing model by the addition of a more generalized inhibition term and the consideration of pH influence on acid dissociation (only undissociated acids enter the cell membrane).

Detailed stoichiometric equations were developed in Papoutsakis (1984) and Dessai et al. (1999), leading to essential contributions to ABE modeling. But the first dynamic kinetic model comprising a detailed metabolic scheme with the inclusion of intracellular intermediates was first published by Shinto et al. (2007). In that work, a kinetic model was built upon detailed ABE metabolic pathway (considering

intracellular metabolites), with 19 rate equations that aim at describing not only inhibitory mechanism (inhibition by glucose and butanol) but also “activatory”. Indeed, previous investigation of the same team (Tashiro et al. 2004) found that addition of butyric acid during solventogenesis resulted in acceleration of butanol production and favors butanol yield. The original model of Shinto et al. (2007) was updated in several improved versions since its publication, that account for the study of xylose utilization (Shinto et al. 2008), or even multiple sources of sugar (Raganati et al. 2015). The latest included some additional modifications like a more general inhibition function and the consideration of the cell’s death term in the presence of butanol. The main limitations of this model rely on the lack of consideration of the pH influence on metabolic switch (only the sugar concentration governs the biphasic shift) and the difficulty associated to the complete estimation of the set of kinetic parameters (Mayank et al. 2013). Besides, no concerns about different types of biomass, or coexisting distinct physiological states of the culture in the bioreactor appear in these models.

Explicit coexistence of different culture subpopulation was first considered in the model developed by Karstens et al. (2016). Concretely, three families are considered (acidogenic, transition and solventogenic), with associated catabolic activities. By means of an experimental cascade of CSTR with appropriate dilution rate, metabolic phases of culture can be separated for modeling, using previously reported kinetic models. Nor sporulating forms neither cell lysis is included, and therefore results presented more divergence in the last part of the train of CSTR (where butanol concentration is higher). Only total biomass was validated experimentally, but the biomass distribution into the three subpopulations was obtained by calculus in each bioreactor. Few details are reported about the kinetics of cell subpopulations, apart from the fact that transition between the three families is mediated by the total concentration of undissociated acids in the broth.

Lastly, in Table 5.1 some reported works dealing with modeling of an ISPR-based process are referred. Park and Geng (1996) reported a fed-batch ABE model coupled to a pervaporation membrane. Based on modified Monod kinetics, their model used a numerical signal based on butyric acid concentration to active (or not) acids production/uptake and/or solvents formation. Mass balances of extracellular metabolites are developed, considering the volume variation in the reactor. Recently, Darkwah et al. (2018) introduced simplified kinetics in ASPEN to simulated batch ABE fermentation coupled to gas stripping .

Table 5.1. Existing dynamic models for ABE fermentation

	Reference	*Kinetics/ Product inhibition*	*Biomass segregation?*	*Mode Flow*	*ISPR*
Unstructured models	(Votruba, et al., 1985) (Yerushalmi, et al., 1986)	Monod / Hyperbolic (butanol)	Yes. Physiologic state (inoculum age)	Batch	No
	(Srivastava & Voleski, 1990)	Monod /Generalized (Luong)	No	Batch	No
	(Napoli, et al., 2011)	Monod /Linear (butanol)	No (vegetative cells)	Continuous	No
	(Park & Geng, 1996)	Monod /Generalized (Luong)	No	Fed-Batch	Pervaporation
	(Karstens, et al., 2016)	Monod /Exponential (butanol)	Yes. Three subpopulations	Continuous	No
	Darkwah et al.2018	Model Votruba et al. 1986			Gas Stripping
Structured models	(Papoutsakis, 1984)	Not done	No	Batch	No
	(Shinto, et al., 2007) (Li, et al., 2011)	Michaelis-Menten / Hyperbolic (substrate and butanol)	No		No
	(Raganati et al. 2015)	Michaelis-Menten /Generalized			
	(Millat et al. 2013)	Michalis-Menten (pseudo-lumped)	Yes. Two pH-dependent phenotypes	Continuous	No

In this work, a dynamic model for ABE fermentation is developed aiming at reflecting the relevant physiological basis of this process. The model will be based over a complete set of electron and mass balanced reactions, considering the main liquid and gas extracellular metabolites (unstructured model). Additionally, an alternative Clostridia cell cycle based on previous experimental work was included in the model. accounting for heterogeneity in the bioreactor (segregated model). Subsequently, *in situ* concomitant liquid-extraction is integrated in the model, considering previously acquired experimental thermodynamic data (Chapter 2).

The novelty of this works relies on the consideration of culture subpopulations quantitative evolution through a batch ABE fermentation, based on experimental data and flow cytometry information (Chapter 4, Tracy et al. 2008). Particularly, the metabolic switch from acidogenesis towards solventogenic phase is triggered by the partial conversion of acid vegetative subpopulation into solventogenic cells.

5.2 Methods

The model was implemented using a combined Matlab/Simulink and Excel framework (Rodriguez et al., 2009), which builds upon a modular and highly flexible methodology to facilitate model developments.

5.2.1 Main assumptions of the model

Dynamic mass balances over aqueous reaction volume were developed for substrate, main extracellular products (butyric and acetic acids, butanol, acetone, ethanol, hydrogen and carbon dioxide) and four subpopulations of clostridia culture (acidogenic, solventogenic, sporulating and dead/lysed cells). While details will be given in further specific sections, general assumptions are listed below:

- The bioreactor behaved as a perfectly mixed system, operated at constant temperature and pressure.

- Glucose is the only limiting substrate, and there is no process limitation by the nitrogen source

- ABE product spectrum is governed by end-product inhibition and culture heterogeneity evolution. Product inhibition is caused by the accumulating undissociated acids (butyric) and solvents (butanol), and their concentrations depend, in turn, on the culture conditions. Undissociated butyric and butanol were also assumed to be, respectively, the triggering factor of solventogenic and sporulating microbial subpopulations.

- Butanol is synthetized from both metabolic routes: acids consumption pathway and directly from the C-subtrate only (Jang et al. 2012).

No mass transfer limitations between phases are considered in biphasic reactor. Thus, in the case of extractive fermentation, equilibrium between organic and aqueous phase is instantaneous through the fermentation time.

As most of kinetic models, it is assumed that changes in metabolite concentrations are fixed by the metabolic network the rate expressions for each biochemical conversion and the transport/transfer phenomena. Therefore, the main structure of the model is expressed in equation system matrix form:

$$\left[\frac{dX_i}{dt}\right]_{i:1:m} = [SM]_{mxn} \cdot [q_r]_{nx1} + [TM]_{mxl} \cdot [q_t]_{lx1} \quad (5.1)$$

The vector on the left-hand side contains the differential equations reflecting temporal changes in the concentration of the m state variables (metabolites, biomass…) considered in the system. The first matrix on the right-hand side is the stoichiometric matrix (SM), and accounts for the n reactions considered to represent the metabolic network. Coefficients in SM are negative or positive according to the number of molecules consumed or generated through each biochemical reaction. The determination of the stoichiometric matrix is based on chemical and biological information and their coefficients result from both mass and electron balances (Rodriguez et al. 2009). The SM is multiplied by the vector containing the reaction rate expressions (q_r), which represents the rate of a given biochemical reaction depending on a several environmental factors (inhibitors, pH…). The two last terms in the right side of the equation (5.1) correspond to the transport matrix (TM) and the transport rate expression vector (q_t). These terms account for equilibria thermodynamics and potential mass-transfer limitations existing between phases. Specific elements referred to concomitantly liquid-liquid extraction of produced metabolites in a biphasic system are included here

5.2.2 Nomenclature

The notation of the main variables and parameters used in the model is presented in the next table of symbols

Symbol	*Definition*
f_{ac}	fraction of glucose uptake into butyric acid during acidogenesis
f_{bu}	fraction of glucose uptake into acetic acid during acidogenesis
f_{s_buoh}	fraction of direct glucose conversion into ethanol during solventogenesis
f_{s_etoh}	fraction of direct glucose conversion into butanol during solventogenesis

Symbol	*Definition*
k_{activ}	activation factor for butyric acid uptake into butanol and acetone
k_d	cell death constant (h^{-1})
k_{lys}	lysis rate constant (h^{-1})
K_{s_ac}	acetic acid half saturation constant (mol S_{buh}/L)
K_{s_bu}	acetic acid half saturation constant (mol S_{ach}/L)
K_{s_glu}	substrate half saturation constant (mol S_{glu}/L)
k_{xa2s}	acidogenic to solventogenic cell transformation rate constant (h^{-1})
k_{xs2sp}	solventogenic to sporulating cell transformation rate constant (h^{-1})
q_{maxA_glu}	maximum specific direct uptake rate of glucose during acidogenesis (h^{-1})
q_{maxSac}	maximum specific uptake rate of undissociated acetic acid into butanol and acetone (h^{-1})
q_{maxSbu}	maximum specific uptake rate of undissociated butyric acid into butanol and acetone (h^{-1})
$q_{maxS\_glu\_d}$	maximum specific direct uptake rate of glucose during solventogenesis (h^{-1})
rA_{ac}	glucose conversion rate into acetic acid during acidogenesis (mol S_{glu}/L h)
rA_{bu}	glucose conversion rate into butyric acid during acidogenesis (mol S_{glu}/L h)
rS_{ac}	undissociated acetic acid conversion rate into butanol and acetone (mol S_{ach}/L h)
rS_{bu}	undissociated acetic acid conversion rate into butanol and acetone (mol S_{buh}/L h)
rS_{buoh}	glucose direct consumption rate into ethanol (mol S_{glu} /L h)
rS_{etoh}	glucose direct consumption rate into butanol (mol S_{glu} /L h)
rX_{a2s}	acidogenic to solventogenic cell type transformation rate (mol X_{act}/L h)
rX_d	cell decay rate (mol X_{act}/L h)
rX_{lys}	lysis rate (mol X_d/L h)
rX_{s2sp}	solventogenic to sporulating cell type transformation rate (mol X_{slv}/L h)
S_{ac}	acetic acid soluble concentration (mol /L)
S_{actn}	acetone soluble concentration (mol /L)
S_{amn}	amonia soluble concentration (mol /L)
S_{bu}	butyric acid soluble concentration (mol /L)
$S_{buh\_critical}$	critical undissociated butyric acid concentration triggering rXa2s (mol /L)
S_{buh_max}	maximal undissociated butyric acid in inhibition function (mol /L)

Symbol	*Definition*
S_{buoh}	butanol soluble concentration (mol /L)
S_{buoh_cri}	critical butanol concentration triggering rXs2sp (mol /L)
S_{buoh_max}	maximal butanol concentration in inhibition function (mol /L)
S_{etoh}	ethanol soluble concentration (mol /L)
S_{glu}	glucose soluble concentration (mol /L)
S_{h2}	hydrogen soluble concentration (mol /L)
S_{ic}	carbone dioxide soluble concentration (mol /L)
S_{lys}	lysed cell material soluble concentration (mol /L)
X_{act}	acidogenic cells concentration (mol/L)
X_d	dead cell concentration (mol/L)
X_{slv}	solventogenic cells concentration (mol/L)
X_{sp}	sporulating cells concentration (mol/L)
$\alpha_{\_bu\_max}$	exponential constant in acid inhibition function
$\alpha_{\_buoh\_ma}$	exponential constant in solvent inhibition function

5.2.3 Stoichiometric matrix

The reactions selected in the model with respective stoichiometric coefficients are presented in Table 5.2. The reaction system is based on the well-known ABE metabolic network, after lumping several intermediary steps to account only for extracellular metabolites. Each reaction is self-activated according to the biomass evolution inside the bioreactor. Thus, dynamic product spectrum is governed by the cell cycle, whose specific transformations appear in the last right five columns of the Table 5.2.

The reactions considered for acidogenic phase account for glucose consumption for acetate and butyrate formation (rAac and rAbu respectively). For sake of simplicity both reactions were combined, assuming constant molar ratio butyrate/acetate (Napoli et al. 2012, Jones and Woods, 1986). Acidogenic biomass yield (Y_{Xact}) was obtained from experimental data (Chapter 4). Indeed, it was considered that biomass growth only corresponds to acidogenic subpopulation at the beginning of the batch fermentation.

The reactions considered for solventogenic phase account for solvent (Acetone, Butanol, Ethanol) production. rSac and rSbu refer to acetic and butyric consumption respectively, together with glucose, to produce butanol and acetone. But butanol (and ethanol) can also be produced in a direct way (Jang et al. 2012), with no participation of the acid loop. In the stoichiometry matrix, rSetoh and rSbuoh refer

to glucose consumption rate to ethanol and butanol direct production respectively. Solventogenic biomass yield (Y_{Xslv}) could not be directly estimated from the data, since both population (acidogenic and solventogenic) were expected to coexist after metabolic switch. Therefore, ATP counting method was applied, assuming that the same acidogenic ratio (mol ATP/mol biomass) is maintained during solventogenesis.

Table 5.2. Stoichiometry Matrix (SM). State variables (1st column) include: aqueous soluble species (S) and biomass families (X). Metabolic reactions are included as well as transformations between cell types.

	rAac	*rAbu*	*rSetoh*	*rSbuoh*	*rSac*	*rSbu*	*rXa2s*	*rXd*	*rXs2sp*	*rXlys*
Sglu	-1	-1	-1	-1	-2.02	-2.03	0	0	0	0
Sac	1.68	0	0.005	0.005	-1	0	0	0	0	0
Sbu	0	0.84	0	0	0	-1	0	0	0	0
Setoh	0	0	1.96	0	0	0	0	0	0	0
Sactn	0	0	0	0	1.00	1.01	0	0	0	0
Sbuoh	0	0	0	0.98	1.50	2.01	0	0	0	0
Sh2	3.26	1.58	0	0	2.00	2.01	0	0	0	0
Samn	-0.19	-0.19	-0.02	-0.02	-0.02	-0.02	0	0	0	0
Sic	1.68	1.68	1.97	1.97	5.01	5.03	0	0	0	0
Slys	0	0	0	0	0	0	0	0	0	1
Xact	0.96	0.96	0	0	0	0	-1	-1	0	0
Xslv	0	0	0.5	0.5	0.5	0.5	1	0	-1	0
Xsp	0	0	0	0	0	0	0	0	1	0
Xd	0	0	0	0	0	0	0	1	0	-1

5.2.4 Clostridia cell cycle

Four Clostridia subpopulation were considered according to their specific metabolic activity: (Figure 5.2) acidogenic and solventogenic cells (Xact and Xslv respectively), sporulating phenotypes (Xsp), and dead cells (Xd). Both Xac and Xslv are growth associated cells (can growth and divide) and were assumed to be the main responsible to acids and solvents (ABE) production respectively. In batch mode, metabolic switch and solvent production is overlaid by sporulation. Xsp integrates all sporulating forms without metabolic activity (forespore, endospore and free spore). Clostridia (cigar-shaped) form was longtime considered the butanol productor phenotype. According to Flow Cytometry data previously discussed (Tracy et al. 2008, Chapter 4), it was instead here assumed to be "transition" form, precursor to sporulation, without specific metabolic activity.

Spontaneous autolysis in batch fermentation has been reported to occur in batch ABE fermentation preceding sporulation (Liu et al. 2015). This phenomenon has also been systematically observed in our assays (Chapter 4), by a sharp decrease in biomass concentration, and the sudden formation of a viscous material (lysin) in the aqueous fermentation broth. In extractive fermentation with Pomace oil, the lysis material was fully extracted into the oil phase. The autolysis was suggested to have an important role related to spore formation (Liu et al. 2015). Thus, the kinetics of dead cells (Xd) decomposition into soluble lysed materials (Slys) was included in the model.

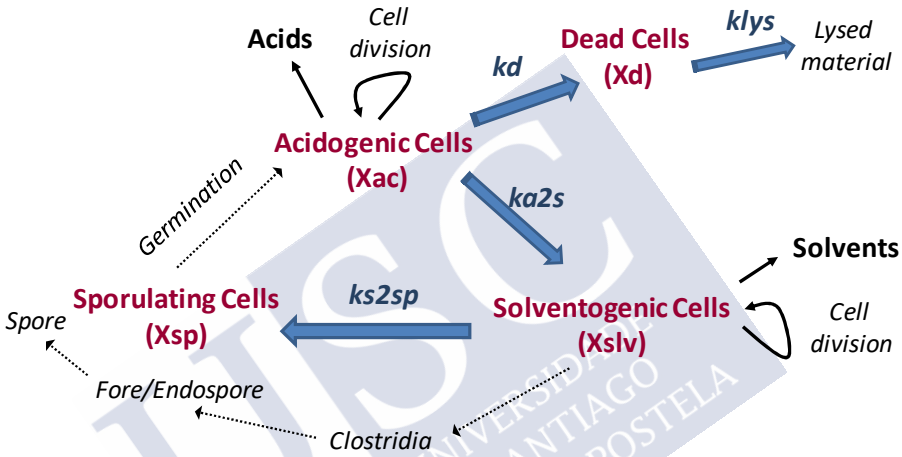

Figure 5.2. Clostridia cell cycle proposal for the model

In previous reported works, a critical threshold concentration of total undissociated acids in the broth was assumed to trigger the switch between acidogenic and solventogenic phases (Monot et al. 1984, Terraciano et al. 1986). Upon this hypothesis, a fraction of the acidogenic population (Xact) is assumed to evolve into solventogenic (Xslv) as an adaptative response to the derived high intracellular acidic conditions. Adapted Xslv cells produce solvents and have (slower) growth. Under the assumption of this model, they derive into sporulating forms (Xsp) as a response to the stress induced by toxic solvents produced (butanol).

The rate of Xact subpopulation evolution ($r_{Xact,}$) is expressed as follows (5.2), where Sig in the second term indicates a physiological signal that switches on when critical concentration of undissociated butyric acid in the broth ($S_{buh,\ critical}$) is reached. The metabolic shift is numerically expressed as a continuous "smoothed" sigmoidal Heaviside function, based on critical acid concentration attained in the broth:

$$r_{Xa2s} = sig \cdot k_{a2s} \cdot X_{act} \quad (5.2)$$

$$S_{ah} \geq S_{ah_{crit}} \quad sig = 1$$
$$S_{ah} < S_{ah_{crit}} \quad sig = 0 \quad (5.3)$$

Based on critical butanol concentration attained in the broth ($S_{buoh\_crit_t}$), the same approach is used to activate the rate of solventogenic population evolving into sporulating family (r_{Xs2sp}):

$$r_{Xs2sp} = sig\_b \cdot k_{xs2sp} \cdot X_{slv} \quad (5.4)$$

$$S_{buoh} \geq S_{buoh\_crit} \quad \text{sig\_b = 1}$$
$$S_{buoh} < S_{buoh\_crit} \quad \text{sig\_b = 0} \quad (5.5)$$

Lastly, the rate of acidogenic cell decay ($r_{Xd,}$) is considered:

$$r_{Xd} = k_d \cdot X_{act} \quad (5.6)$$

5.2.5 Reaction and transfer rate expressions

The rate of glucose consumption during acidogenesis (r_{A_Glu}) is expressed as follows:

$$r_{A\_Glu} = q_{maxA\_Glu} \cdot \frac{S_{Glu}}{K_{S_{Glu}} + S_{Glu}} \cdot Inhib_{ah} \cdot Inhib_{solv} \cdot X_{act} \quad (5.7)$$

Where q_{maxA_Glu} represents the maximum specific uptake rate of glucose for butyric and acetic acid production, and K_{SGlu} is the substrate half saturation constant. $Inhib_{ah}$ and $Inhib_{solv}$ are the total undissociated acid and solvent inhibition terms. The equation (5.7) is a linear combination of equations (5.8) and (5.9), which are associated with the butyric and acetic formation respectively.

$$r_{A\_ac} = f_{ac} \cdot r_{A\_Glu} \quad (5.8)$$

$$r_{A\_bu} = f_{bu} \cdot r_{A\_Glu} \quad (5.9)$$

$$f_{ac} + f_{bu} = 1 \quad (5.10)$$

The rate of glucose consumption during solventogenesis to be directly into alcohols – butanol and ethanol - ($r_{S\_Glu\_d}$) is expressed in an analogous way:

$$r_{S\_Glu\_d} = q_{maxS\_Glu\_d} \cdot \frac{S_{Glu}}{K_{S_{Glu}} + S_{Glu}} \cdot Inhib_{ah} \cdot Inhib_{solv} \cdot X_{slv} \quad (5.11)$$

Where $q_{maxS\_Glu\_d}$ represents the maximum specific direct uptake rate of glucose during solventogenesis. The equation (5.11) is assumed to englobe both the direct butanol (5.12) and ethanol (5.13) formation.

$$r_{S\_buoh} = f_{buoh} \cdot r_{S\_Glu\_d} \quad (5.12)$$

$$r_{S\_etoh} = f_{etoh} \cdot r_{S\_Glu\_d} \quad (5.13)$$

$$f_{etoh} + f_{buoh} = 1 \quad (5.14)$$

Solventogenic biomass also participates in acid uptake reactions. The formation of acetone is performed by the enzymes acetoacetyl-CoA-transferase and acetoacetate decarboxylase in two sequential steps. These enzymes have strong pH-dependence kinetic activity (Millat et al. 2013). The uptake of the acids into the respective acetyl and butyril-CoA pools, happens via the same enzyme acetoacetyl-CoA-transferase. Therefore, these reactions (acetone formation and acids uptake) are coupled. In this model, they are represented by r_{S_ac} (rate of undissociated acetic acid consumption into acetone and butanol), and r_{S_bu}(rate of butyric acid assimilation into butanol and acetone):

$$r_{S\_ac} = q_{maxS\_ac} \cdot \frac{S_{Glu}}{K_{S_{Glu}} + S_{Glu}} \cdot \frac{S_{ac}}{K_{Sac} + S_{ac}} \cdot Inhib_{solv} \cdot X_{slv} \quad (5.15)$$

$$r_{S\_bu} = q_{maxS\_bu} \cdot \frac{S_{Glu}}{K_{S_{Glu}} + S_{Glu}} \cdot \frac{S_{buh}}{K_{S_{bu}} + S_{buh}} \cdot F_{activ\_buh} \cdot Inhib_{solv} \cdot X_{slv} \quad (5.16)$$

$$F_{activ\_buh} = 1 + k_{activ} \frac{S_{buh}}{S_{buh\_crit}} \quad (5.17)$$

In previous expressions, q_{maxS_ac} and q_{maxS_bu} reflect maximum specific uptake rate of undissociated acetic and butyric into butanol and acetone, and $K_{sac,}$ and K_{Sbu} are the undissociated acids half saturation constant in Monod based formulations. Butyric acid uptake has been reported to be boosted with increasing butyric acid concentration (Tashiro et al. 2004, Shinto et al. 2007). An activation factor (Factiv_buh) is included in (5.16) to account for this effect.

Each reaction rate in ABE fermentation model is governed by product inhibition. Two general microbial growth inhibition functions (Luong et al. 1989) were first included, accounting respectively for the inhibition due to undissociated acids (Inhib_ah) and to the solvents (Inhib_solv). It was deduced from the calibration data that acid and solvent inhibition are respectively governed by undissociated butyric and butanol. Thus, the following inhibition expressions were simplified:

$$Inhib_{buh} = 1 - \left(\frac{S_{buh}}{S_{buh\_max}}\right)^{\alpha_{buh\_max}} \quad (5.18)$$

$$Inhib_{ah} = Inhib_{buh} \quad (5.19)$$

$$Inhib_{buoh} = 1 - \left(\frac{S_{buoh}}{S_{buoh\_max}}\right)^{\alpha_{buoh\_max}} \quad (5.20)$$

$$Inhib_{solv} = Inhib_{buoh} \quad (5.21)$$

Gases produced during ABE fermentation are transferred into gas phase, contributing to total pressure composition inside the bioreactor. Liquid-gas transfer expression used in this model are presented below:

$$r_{TrSH2G} = K_{LA} \cdot (S_{H2} - H_{H2} \cdot P_{H2}) \quad (5.22)$$

$$r_{TrSCO2G} = K_{LA} \cdot (S_{CO2} - H_{CO2} \cdot P_{CO2}) \quad (5.23)$$

Where gas-liquid mass transfer rate constant is expressed as K_{LA} (h^{-1}), H denotes the Henry coefficient and Pi is the partial pressure of the I component in the bioreactor gas phase.

When in situ liquid extraction is applied, transfer of aqueous metabolites (butanol, acetone and ethanol, and undissociated acids) inside the extractant phase is also predicted with the model. Mass transfer rate constant K_{LL} is fixed high enough to neglect the transfer limitation (experimental information in Chapter 3, Chapter 4). Partition coefficient for metabolite into the extracting agent are experimentally obtained in Chapter 2. For instance, butanol transfer rate into the organic phase is calculated as follows:

$$r_{TrSbuohLL} = K_{LL} \cdot \left(K_{eq_{buoh}} \cdot S_{buoh} - E_{buoh}\right) \cdot \frac{V_{ext}}{V_{aq}} \quad (5.24)$$

In previous expression, butanol concentration in the organic (extractant) phase is expressed as E_{buoh} (mol/L_{org}), and V_{ext} and V_{aq} refer respectively to extractant and aqueous phase volume inside the bioreactor.

5.2.6 Parameters estimation

A two-step procedure was applied for parameter estimation. Firstly, some of the values were obtained by combining literature data and a heuristic search to match own experimental data. As an instance, maximal specific glucose uptake rate for acidogenic production can be obtained from initial batch experimental data as follows:

$$q_{maxA\_Glu} = \frac{\mu_{\max\_Xac}}{Y_{Xac/Glu}} \tag{5.25}$$

Where μ_{max_Xac} is estimated from maximal slope of initial dry cell weight evolution over time.

In a second step, a data-versus-simulation error objective function (FO) was defined in order to estimate the remaining unknown parameters – those presenting higher variability - by error minimization. The function used was the squared differences between the prediction from the model and experimental data used for calibration. Parameter optimization was applied upon the experimental data published in Monot et al. (1984), which consist of four data sets at different fixed pH values using the strain *Clostridium acetobutylicum* ATCC824, and with initial glucose concentration of 53 g/L.

$$FO = \left[y_{exp}(t) - y_{model}(t)\right]^2 \tag{5.26}$$

Parameter values related to acid inhibition and critical butyric acid concentrations that trigger the metabolic switch were estimated by this error minimization method.

To confirm the validity of the estimated parameters, the calculated time courses were compared with our experimental data obtained in a free-pH run, at initial glucose of 85 g/L (Chapter 4).

5.3 Results

5.3.1 Optimized model parameters

Optimized kinetic parameters of rate expressions presented previously are compiled in Table 5.3.

Tabla 5.3. Kinetic parameters

Parameter	Value	Unit
q_{maxA_glu}	0.35	mol S_{glu}/L/mol X_{act}
f_{ac}	0.40	[]
f_{bu}	0.60	[]
K_{s_glu}	0.02	mol S_{glu}/L
K_{s_ac}	0.05	mol S_{ac}/L
K_{s_bu}	0.03	mol S_{bu}/L
$q_{maxS\_glu\_d}$	0.23	mol S_{glu}/L/mol X_{slv}
f_{s_buoh}	0.80	[]
f_{s_etoh}	0.20	[]
q_{maxSac}	0.20	molS_{ac}/mol X_{slv}.h
q_{maxSbu}	0.20	molS_{bu}/molX_{slv}.h
k_{activ}	2	[]
k_d	0.10	h^{-1}
k_{xa2s}	10.00	h^{-1}
k_{xs2sp}	0.01	h^{-1}
k_{lys}	0.40	h^{-1}
S_{buh_max}	pH dependent	mol S_{buh}/L
$\alpha_{\_bu\_max}$	0.70	[]
S_{buoh_max}	0.19	mol S_{buoh}/L
$\alpha_{\_buoh\_max}$	1.00	[]
$S_{buh\_critical}$	pH dependent	mol S_{buh}/L
$S_{buoh\_critical}$	0.07	mol S_{buoh}/L

Both the inhibition coefficient (S_{buh_max}) and the metabolic switch trigger threshold (S_{buh_crit}) presented variability with external controlled pH, as shown in Figure 5.3. A

polynomial function capturing the variation of the external undissociated butyric related parameters was used in the model (Figure 5.3).

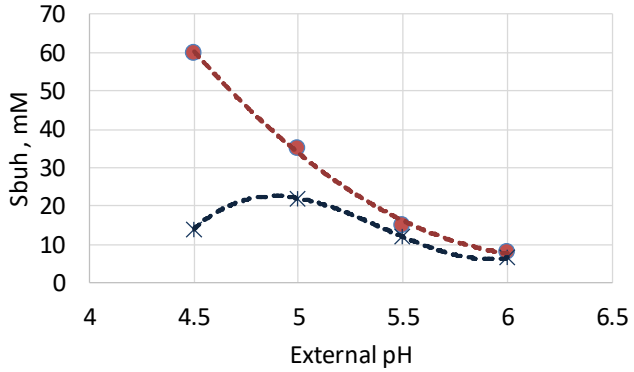

Figure 5.3. External pH-dependency of Sbuh_max (circle) and Sbuh_crit (asterisk)

5.3.2 Batch fermentation at fixed external pH (calibration)

The model was developed based on the simplified metabolic network of *C. acetobutylicum*, englobing representative mass-balanced reactions that are activated by specific Clostridia subpopulations. Thus, culture heterogeneity governs the ABE metabolism, and it is, in turns, dependent on changing environmental conditions. Particularly, pH has been identified as a key variable in the orchestration of biphasic metabolism ABE behaviour (Millat et al. 2013).

Literature data set at fixed external pH varying from 4.5 to 6 (Monot et al. 1984), were used to calibrate the developed model. Comparisons between experimental and simulated results on main extracellular metabolites (glucose, ABE and acids) are depicted in Figure 5.4. At acidic conditions (Figure 5.4 A), solventogenic metabolism predominates, with minimal acid formation and solvent production is triggered from the beginning of time course. With increasing pH, the acid to solvent ratio increases, and this trend is well captured by the model as it can be seen in Figure 5.4. At pH 6 (Figure 5.4D), solventogenic production is minimal, and total butyric acid in the broth reaches more than 12 g/L. This is expected from acidogenic fermentations, controlled at pH close to neutrality, as stated in Monot et al. (1984). However, acidogenic fermentations usually do not present any metabolic switch,

but continuous acid production. Instead, we believe that experimental data in Figure 5.4D revealed an “acid crash”, since an effective switch indeed occurred, just preceding a metabolic activity cessation (Maddox et al. 2000). Overestimations of both solvent production and glucose consumption after metabolic switch would be then attributed to different phenomena associated to acid crash (i.e. high intracellular accumulation of formic acid, Wang et al. 2011) that could not be predicted by the model.

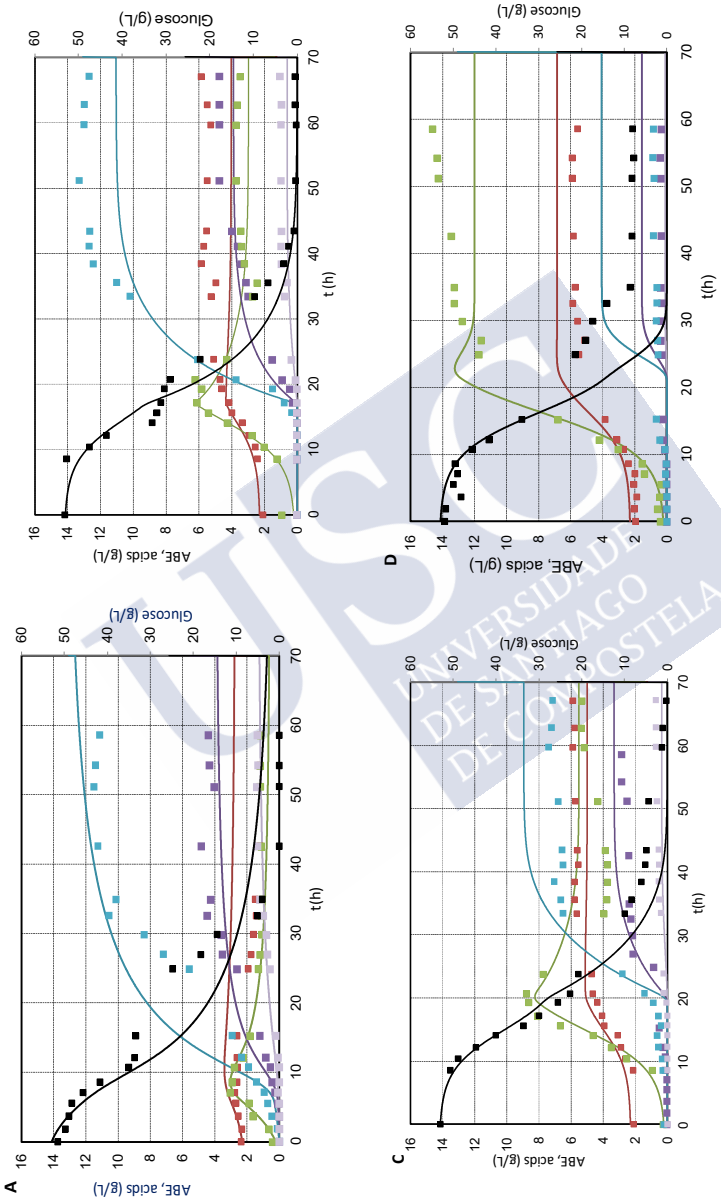

Figure 5.5. Comparison of experimental data from four independent experiences reported in Monot et al. (1984) (symbols) and simulation (solid lines). Glucose (black), butanol (blue), acetone (violet), ethanol (mauve), acetic acid (red), butyric acid (green). External controlled pH: A) 4.5, B)5, C) 5.5 and D) 6

In our model, the product spectrum prediction results appear to rely on the microbial subpopulations dynamics, which are presented in Figure 5.5. Although only experimental total biomass concentration is available to compare with the model results, the model provides additional information regarding the possible Clostridia cell culture heterogeneity and evolution. The modeled microbial growth fit well with the experimental data at pH values from 4.5 to 5.5.

The time course of total and specific biomass exhibited further features that support our hypothesis of the existence of a growth-associated solventogenic subpopulation. At lower external pH values (4.5 and 5), the biomass concentration continued to increase well after solvent production was effectively triggered. In these cases, a metabolic shift occurred far from both acid and solvent inhibition levels. Thus, the new "adapted" solventogenic population could divide and grow (accompanying solvent production) before the activity was fully ceased. Therefore, both acidogenic and solventogenic cells were responsible of total microbial growth.

Solventogenic cells started to sporulate under external stress. In our model, this evolution is triggered by critical aqueous butanol in the broth (supported by our experimental data presented in Chapter 4). Therefore, sporulating subpopulation (which comprises forespores, endospores and free spores) increased with higher butanol concentration in the aqueous broth (Figures 5.4 and 5.5).

Spontaneous lysis of dead biomass was included in the model and was assumed to occur after the metabolic switch, preceding sporulation. Nevertheless, at acidic conditions (Figure 5.5A), a biomass concentration decrease is only appreciated at the end of the experiment. On the contrary, close to neutrality (Figure 5.5 D), lysis seems to be accelerated respect to model predictions. One possible explanation is that cellular death might be enhanced at more neutral conditions when undissociated acid inhibition is stronger, thus anticipating lysis phenomena. However, in our model, cell's death constant (k_d) was fixed constant, independently of pH conditions. However, this cannot be claimed.

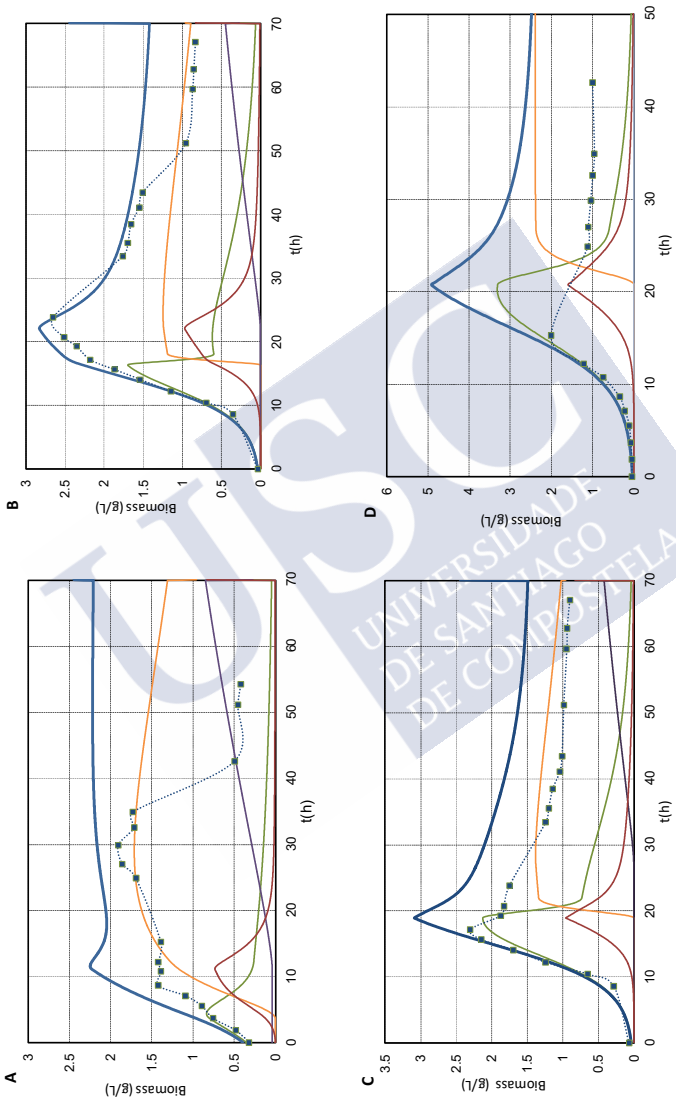

Figure 5.6. Comparison of experimental data from four independent experiences reported in Monot et al. (1984) (symbols) and simulation (solid lines). Total biomass (blue), acidogenic (green), solventogenic (orange), sporulating (violet), dead cells (red). External controlled pH: A) 4.5, B)5, C) 5.5 and D) 6

5.3.2 Model validation in batch fermentation at uncontrolled pH

Uncontrolled pH batch fermentation carried out in a 2L BIOSTAT STR (Chapter 4) was used to validate the developed model. The same strain (*C. acetobutylicum* ATCC824), protocol and synthetic media composition as described above were applied, except for the glucose concentration (83 g/L here against 50 g/L in reported data of Monot et al. (1984)). The experimental pH profile measured was tracked dynamically with time as an input variable to the model. At the time of metabolic switch, the minimum pH value is reached at approximatively 4.6 (Chapter 4). The inhibition and triggering parameters corresponding to pH 4.5 were therefore applied.

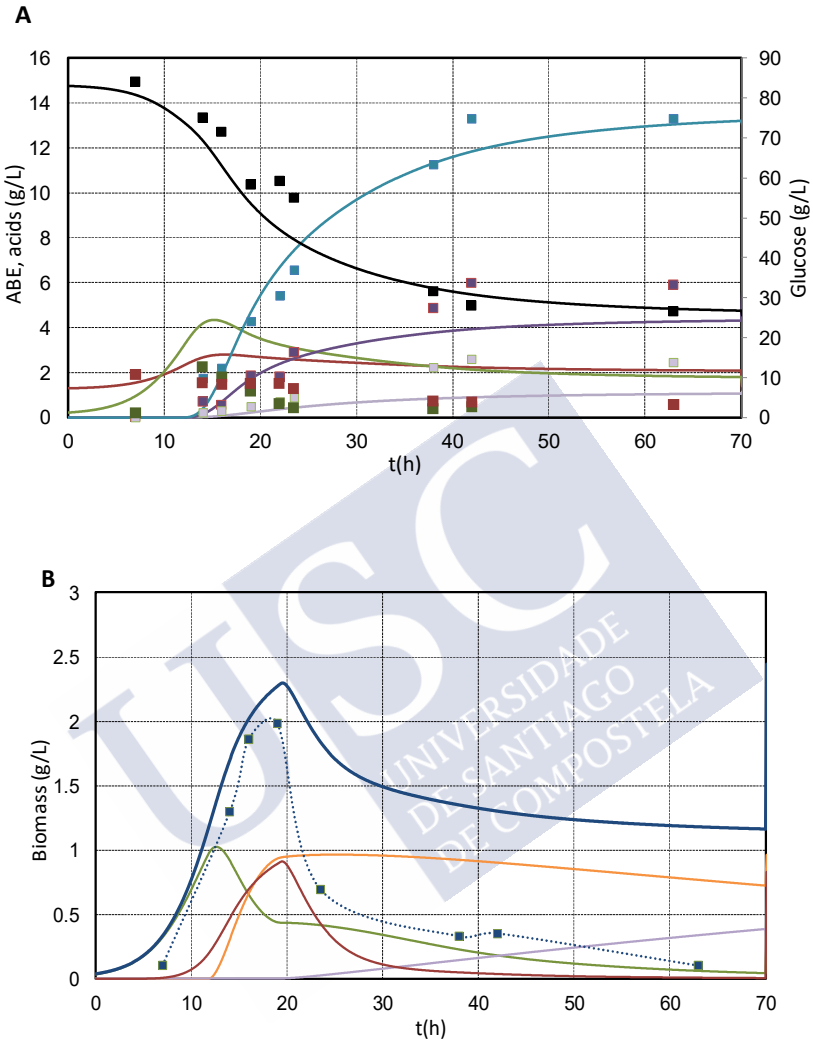

Figure 5.6. Model validation with the experimental data described in Chapter 4. Temporal behavior of A) Extracellular metabolites, B) Biomass concentration. Color code is the same as in Figure 5.5 and 5.6). In A) Glucose (black), butanol (blue), acetone (violet), ethanol (mauve), acetic acid (red), butyric acid (green).In B) Total biomass (blue), acidogenic (green), solventogenic (orange), sporulating (violet), dead cells (red).

The simulated profiles for both glucose and butanol fit well with experimental data (Figure 5.6 A). Microbial growth increased up to 20h of fermentation run, when the solventogenic phase was well advanced. This is well captured by the model, and in agreement with simulated microbial population dynamics (Figure 5.6 B). This

provides information about the respective relative contributions of acidogenic and solventogenic groups. After 20h of fermentation, the sporulating activity was initiated and maintained up to the end of the fermentation. The metabolic activity ceased when the aqueous butanol exceeded 12 g/L, even with a remaining glucose concentration higher than 20 g/L.

For this fermentation run, some degree of quantitative distribution in microbial heterogeneity was determined by the application of flow cytometry (Chapter 4). Although acidogenic cells could not be distinguished from solventogenic ones, the total vegetative subpopulation could be quantified and compared against total sporulating forms (forespore, endospore and free spores). Model predicted are presented against experimental in Figure 5.7. Quantification of the different cell types in the inoculum was available and was introduced at an input to the simulation. Only vegetative cells (acidogenic and solventogenic) constituted the bioreactor culture at around 20h of fermentation, after that their contribution decreased in favor of the sporulating cells. The unexpected increase of sporulating cells at around 20h was discussed in Chapter 4. The bulk of inactive lysed cells (more propitious to form aggregates, higher size, higher granulometry) may have assigned to the sporulating group at that point, as also observed by Tracy et al. (2008).

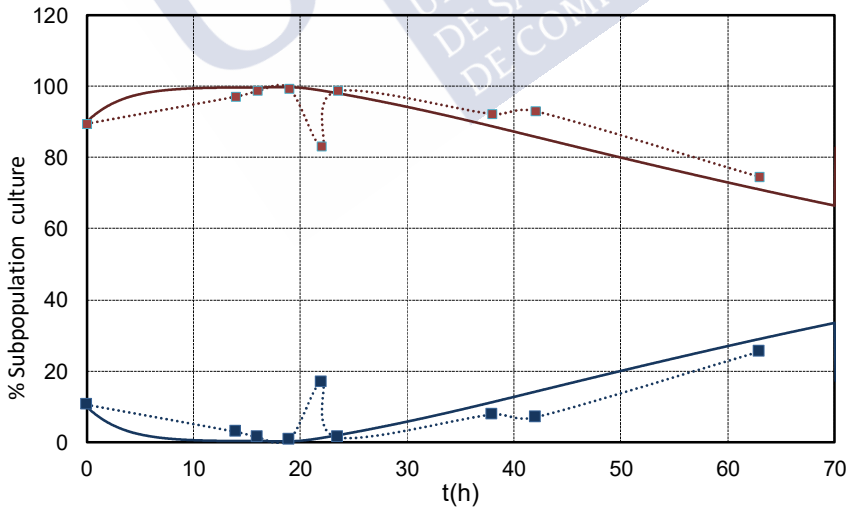

Figure 5.7. Simulated subpopulation cultures (continuous line) versus experimental Flow Cytometry data (symbols with dashed lines). Growing cells (red), sporulating cells (blue)

5.3.3 Simulated extractive fermentations

In situ extractive fermentation was applied in a 2L BIOSTAT STR, with two solvents presenting different levels of biocompatibility (Chapter 4): a low capacitive vegetable oil (KBuOH =0.6 g/g), and a C12-based Guerbet alcohol, 2-butyl-1-octanol, which presented stronger affinity for butanol (KBuOH = 6.76 g/g), but also higher polarity (logP ~4). These solvents were present at the beginning of the batch experiences; thus, extraction was a priori effective in all phases of the fermentation, and mass transfer functions were applied to all metabolites (ABE, acids) based on experimental partition coefficients (Chapter 2).

Figure 5.8 compares experimental and simulation data for vegetable oil extractive fermentation. Theoretically, toxic product removal from the aqueous broth means higher volumetric productivity, since inhibition function is alleviated over standard fermentation case. This increase in productivity was predicted by the model and simulated total butanol concentration (referred to aqueous phase) reached 15 g/L in 40h. However, experimental data for extractive fermentation with Vegetable Oil did not increase standard case productivity, and butanol production is slowed down from 20h of the run (Chapter 4).

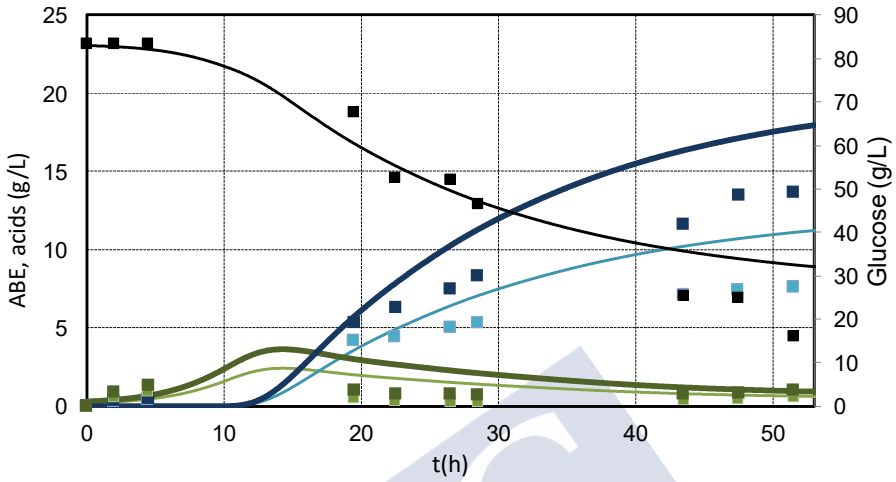

Figure 5.8. Simulated (continuous line) versus experimental (symbols) data in extractive fermentation with Vegetable Oil. A). Total butanol (dark blue), aqueous butanol (light blue), total butyrate (dark green), aqueous butyrate (light green), glucose (black)

Several hypotheses can be emitted to support these observations. Even if Vegetable Oil is expected to have full biocompatibility towards Clostridia, toxicity effects at the interphase between the aqueous and extracting phase might have not been avoided. These effects have been postulated to be independent of the solvent polarity, and to be more linked to physical phenomena (steric and diffusion limitations around the cell membrane, clogging…) (Salter et al. 1995, Daugulis 1997). This behavior cannot be anticipated with the current version of the model. Additionally, over partial pressure of fermentation gas (particularly carbon dioxide) might have been created in the aqueous broth during extractive fermentation, generating additional inhibitory effect that have not been captured by the model. These speculations are complementary and do not exclude others qualitative observations. For instance, lysed material was visually fully extracted into the vegetable oil during batch fermentation, and, possibly linked to this (Liu et al. 2015), sporulating level was minimal during this fermentation (Chapter 4). If spontaneous autolysis presents a biological role that aims at guarantee cell survival, removing lysed material might have negatively affected the fermentation.

Differences between simulated and experimental data were more strongly marked in the case of 2B1O based extractive fermentation (Figure 5.9). Thank to high

extraction capacity of 2B1O, butanol was effectively removed from the broth and aqueous concentration was kept under 5 g/L through the whole run. Neglecting solvent polarity and its potentially negative influence on ABE metabolism, this implies higher reaction rates, as predicted by the model (Figure 5.9) However, productivity is degraded, and metabolic activity strongly affected in the fermentation carried out in 2L STR (Chapter 4). While model predicted glucose depletion at 30h of fermentation and butanol production at high rate (up to 16 g/L), experimental values showed slow (but continuous) glucose consumption and solvent production rates. This corroborates the existence of strong toxicity effects that could not be predicted by the current version of the model.

Solubility of polar 2B1O is higher than that of vegetable oil, and dissolved molecules of 2B1O could attain more easily suspended Clostridia cell membrane. Still, compared to STR, experimental data obtained in 250-ml sealed flask (Chapter 3) presented less marked difference against simulated trends (Figure 5.9 B). In fact, after an increased lag time, cells seem to have adapted to new environmental conditions and glucose was depleted (as predicted by the model). Mainly biphasic toxicity was affected when passing from sealed flask to bioreactor, since both phases were already at equilibria conditions in flask bottles (Chapter 3). This indicates that complex phenomena at the aqueous-organic interphase, that have not been considered in the model, might strongly affect the ABE metabolism in extractive fermentation with 2B1O. At the end of the fermentation in sealed flask (Figure 5.9 B), total simulated butanol in underestimated by 23%. This is in concordance with higher experimental yield observed in the presence of 2B1O, and the origin of this change has not been captured by the current model.

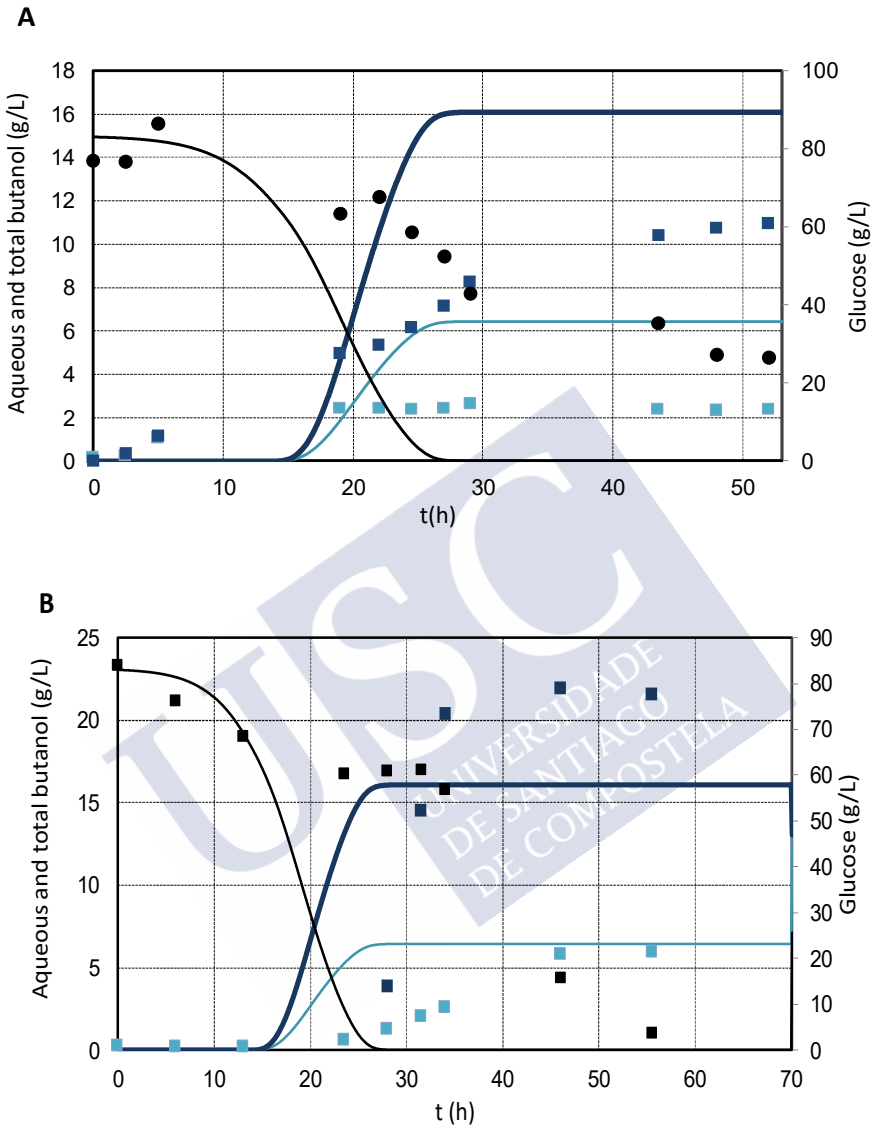

Figure 5.9. Simulated (continuous line) versus experimental (symbols) data in extractive fermentation with 2Butyl1Octanol. A). Total butanol (dark blue), aqueous butanol (light blue), glucose (black). Experimental data obtained in A) 2L-STR; B) 250 ml flask bottle

The application of the model on ABE extractive fermentation did not allow to reproduce experimental data obtained with solvents of different nature. The differences were even more pronounced with 2B1O, which presents lower

biocompatibility towards Clostridia. The observed lack of reliability prevented us to the use of this model on further ABE extractive simulation studies. This analysis confirmed that solvent-ABE metabolism interactions go further than liquid-liquid transfer of metabolites during the fermentation.

5.4 Discussion and conclusions

ABE fermentation is achieved by intrinsically heterogeneous Clostridia populations as it has been supported by experimental data over the last decades (Schuster et al. 2000, Tracy et al. 2008, Patakova et al. 2019). In continuous cultures with suspension cells, this is more easily evidenced by the cyclic oscillations in the levels of acids and solvents (Maddox et al.1989, Tashiro et al. 2005). In standard batch operation, high toxic butanol production and glucose depletion are concomitant, preceding sporulation, and there has been a typical association between solventogenesis, stationary phase and sporulation. However, the role of different cell types co-existing in the culture has been extensively discussed in literature and a simple connection between cell types and fermentation products might not have been well established yet. For instance, the application of flow-cytometry techniques to batch ABE fermentation revealed the existence of a growth associated cell phenotype responsible to butanol production (Tracy et al. 2008). This is supported by our data in Chapter 4 and challenges the long-standing notion of stationary solventogenic state.

Despite experimental evidence, the culture heterogeneity has longtime not been considered in more than 30-year history of ABE fermentation modeling. Millat et al (2013) developed a first dynamic model based on two coexisting microbial growing populations (acidogenic and solventogenic). Their model revealed that the external pH-induced metabolic shift in continuous culture with *Clostridium acetobutylicum* was governed by the switch of these two families. These authors demonstrated that population dynamics might play a key role in the pH-induced metabolic switch of *C. acetobutylicum*. Nevertheless, in continuous culture dynamic features of growth and reaction rates are impacted by the dilution rate (cells wash out). Moreover, critical concentration of solvents is not attained, and consequently sporulation is hardly ever observed in normal conditions.

In the present work, inspired by experimental data, we introduced the hypothesis of a growing solventogenic population into a batch dynamic ABE fermentation model. Complex ABE metabolic network was reduced into a group of representative and well-balanced (mass, electron) biochemical reactions, and each

reaction is self-activated by specific cell population. These reactions include acidogenic glucose consumption into acids, acids (butyric and acetic, separately) assimilation into solvents (butanol, acetone) and direct ethanol and butanol production from glucose. Particularly, the stoichiometric matrix reflected the two different pathways contribute to butanol rate production, direct pathway from glucose and acid uptake (Jang et al. 2012).

In the proposed model, there is a heterogeneous culture inside the bioreactor, consisting of several specialized subpopulations. The product spectrum dynamics will depend on the evolution of this multi phenotypic population. Two active growth-associated cell families were considered: acidogenic and solventogenic, and a latent inactive sporulating subpopulation was also considered. The main assumption subjacent is that culture heterogeneity furnishes Clostridia microorganism self-regulation possibilities to adapt the best and survive under environmental stress. Therefore, transitions between phenotypes respond to key biological role, and triggering factors might be well identified. For instance, the evolution of acidogenic into solventogenic phase would be at the origin of the commonly observed metabolic switch. It has been postulated to be a "detoxification" mechanism when intracellular pH become too low (Jones and Woods, 1986). Also, sporulation begins with the partial conversion of the solventogenic cells into resistant sporulating forms when level of toxic product is high in the medium. Again, this cell transition can be seen as a survival strategy of the heterogeneous culture as a whole.

The proposed model adequately describes the culture inhibition and the dynamic behavior of all the extracellular metabolites in a batch culture with both free and controlled pH level, including unconventional acidogenic fermentation (pH 6).

Most of existing batch dynamic models are insensitive to pH variations in the culture (Yerushalmi et al. 1986, Srivastave and Volesky, 1990, Shinto et al. 2007). However, it is well accepted that pH is the key parameter orchestrating metabolic state in ABE fermentation. On the other hand, some of the model only include the inhibitory role of butanol (Shinto et al; 2007), while butyric and acetic acids might have stronger inhibitory effect (Yang and Tsao 1994). In fact, the culture pH usually crosses the pKa of the butyric acid (4.82), and it has been postulated that the intracellular conversion into solvents is a protective strategy to avoid back diffusion of undissociated acids (Millat et al 2017). Lastly, some literature models associate the metabolic switch to sugar depletion, while most of sugar is converted during the solventogenic phase (Shinto et al. 2007)

In the model developed in this work were expressly distinguished two distinct inhibition functions accounting for the presence of undissociated acids and solvent.

For the calibration data set (Monot et al. 1984) only undissociated butyric and butanol resulted to govern inhibition contributions. Moreover, undissociated butyric acid in the broth was identified as the critical factor triggering the solvent production phase. Nevertheless, the value of these critical values for undissociated acid in the aqueous broth (inhibition and triggering) were found to vary sensitively with external controlled pH (Figure 5.4). Thus, acid inhibition is numerically stronger in this model at close to neutrality conditions (pH6), while the solventogenesis is triggered later at pH 5.

Actually, metabolism is regulated by intracellular conditions, and, from a physiological point of view it would be better to refer to them. It has been reported that *C. acetobutylicum* is unable to maintain a constant intracellular pH, but instead, the cell manages to keep a ΔpH of between 0.9 to 1.3 when external pH varies from 6 to 4.5 (Gottwald and Gottschalk 1985; Jones and Woods 1986, Terraciano and Kashket 1986). Thus, changes in external pH might influence differently the total internal acid concentration. Considering the permeability of the cytoplasmic membrane to the undissociated acids, the ΔpH gradient has been postulated to be maintained by the action of an ATP-dependent proton extrusion mechanism (Gottwald and Gottschalk, 1985). Huang et al. (1985) studied the involvement of the proton motive force /pmf) in *C.acetobutylicum* and concluded that ΔpH electrical contribution is maximal at acidic external conditions (pH 4.5). Those works suggested that total cumulative internal dissociated acid concentrations might activate the culture shift, and not undissociated acid in the broth as suggested by Monot et al. (1984). Our model also supports this view. In fact, under the assumption that uncharged acid can freely permeate through the membrane, total internal butyric acid was estimated in the calibration data set, from broth undissociated and ΔpH managed by the cell at fixed external pH (Huang et al. 1986). The calculated internal concentration presented much lower variation that the external corresponding concentration by the moment of metabolic shift.

Senger and Papoutsakis (2008) introduced the concept of the specific proton during both phases of ABE fermentation in a structural model using flux balance analysis. According to this model, acid and solvent producing cells significantly differ in their proton fluxes. In addition, medium pH in only properly predicted considering a net decrease in proton secretion (per weak acid efflux) form the beginning to the end of the vegetative growth and the existence of multiple states of proton flux states. That is why, interestingly, the authors suggested the existence of several distinct metabolic phenotypes in that papers.

All these considerations prompt us to think that acid inhibition phenomena and metabolic shift triggering might be predicted by proper consideration of the ΔpH effects in further ABE model evolutions. Moreover, solvent butanol inhibition and

its effect on membrane and fluidity has also been previously quantified in term of ΔpH reduction and abolition (Huang et al. 1986, Terraciano and Kashket 1986). The addition of butanol or other uncouplers to the medium during acidogenic phase was postulated to inhibit the ATPase related functions, and then to reduce ΔpH, accelerating solvent production (Monot et al. 1984).

Simulated predictions of extractive fermentation did not reproduce experimental data, and difference were marked with 2B1O as extracting agent. It is speculated that associated phenomena in these fermentations go beyond physical transfer of metabolites between aqueous and organic phases. Instead, presumably complex solvent toxicity effects on Clostridia cell cycle might not be ignored. For instance, premature triggering (with respect to external acid concentration) was systematically observed with extractive fermentation in the presence of 2B1O (Chapter 3). Therefore, including in further model version the intracellular response through variation of trans membrane gradient of pH might help to explain the altered response of Clostridia in biphasic fermentation with polar solvents.

Furthermore, product spectrum and particularly butanol to acetone ratio dependence on intracellular pools of ATP and NAD(P)H have been investigated (Girbal et al. 1995) and might also be considered in future model proposal. Particularly, high levels of both intracellular ATP and NAD(P)H were correlated to alcohologenic metabolism (low acetone produced). Considering this aspect in, and assuming ATPase related function to be interfered by the presence of 2B1O in extractive fermentation, the systematic enhancement of butanol yield (over acetone) in these fermentations might be explained by further version of the model.

The transition from acidogenesis to solventogenesis is accompanied by considerable changes in cellular composition (transcriptome, proteome and metabolome) that are hardly explained by simple kinetic regulations, as inhibitory effects of metabolites or Monod expressions. It has been recently stated that ABE fermentation network is more complex than thought and understanding of two the regulatory mechanism of the two metabolic states remains incomplete (Millat et al ,2017). Liao et al. (2015) reported that at least 245 genes are differentially expressed during phase transition. But unexpectedly, Patakova et al. (2019) found, by transcriptomic profiling, that the main change in gene expression was linked to sporulation triggering. All these interactions should be progressively introduced in upcoming works, in order to let mathematical model, contribute to the improvement of our comprehension of clostridial ABE fermentation.

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6. A solvent-based techno-economical evaluation

Product concentration in Acetone Butanol Ethanol (ABE) fermentation can be significantly enhanced by integrating in situ liquid-liquid extraction. Butanol yield might be also increased in the presence of specific organic solvents. The purpose of this work is to quantify the subsequent economic improvement of the overall process. Hybrid simulations using Excel 2013 and ASPEN Plus v7.3 based on experimental data were performed to provide information about the energy requirements and economics of the ABE extractive fermentation process. Four scenarios, based on two different extractants (2-butyl-1-octanol, 2B1O, and vegetable oil, VO) applied in batch or fed-batch operation, were compared with the conventional process. Total energy demand decreased in all extractive configurations, and VO-based fed-batch resulted in the greatest energy savings of 61%. However, the highest profit increase was achieved with the 2B1O in fed-batch mode, reducing minimum butanol selling price by 29% reduction over base case. For this scenario, simulations indicated more than 80% wastewater reduction, along with 34% savings in raw materials. Besides, a sensitivity analysis of the feedstock demonstrated that the relative profitability of the cases might be altered. It was found that below a sugar price cutoff (200 €/t) the VO scenario becomes feasible.

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OUTLINE

6.1 Introduction

Butanol is a bulk chemical used as a feedstock for petrochemistry, and with multiple direct applications. When biobased, it is also considered as a potential advanced biofuel, with higher energy content and lower hygroscopicity than ethanol (Green, 2011). Butanol can be produced via Acetone-Butanol-Ethanol (ABE) fermentation from renewable resources. ABE fermentation was run commercially until the mid-20th century, when it was progressively overpassed by cheaper petrochemical process (Jones & Woods, 1986). Currently, oil prices volatility and environmental concerns enhanced the reestablishment of ABE fermentation in some countries like China (Xue et al., 2017) or Brazil (Mariano et al., 2013), and prompted research community into the revitalization of this historical process. Low theoretical yields and quick accumulation of toxic end-products in the fermentation broth, specially butanol, are the main intrinsic limitations of the ABE fermentation. As a result, mixtures produced by ABE fermentation contain up to 20-30 g/L of alcohol, leading to high recovery costs and large volumes of wastewater (Green, 2011), (Durre, 2011). The negative effects of end-product inhibition can be mitigated by the application of an In Situ Product Recovery (ISPR) technology, which removes toxic butanol from the culture broth as soon as it is produced, maintaining the culture environment concentration below the toxic threshold. Among the different ISPR options that have been applied to ABE fermentation at laboratory level, liquid-liquid extraction (LLE) has been reported to be one of the most energy-efficient alternatives (Leland, 2008) (Qureshi et al 2005) (Salemme et al. 2016).

The benefits of ABE integration with liquid-liquid extraction have been the object of plentiful publications. Some of them focus on experimental results, demonstrating enhancements in productivity or final concentration (Barton and Daugulis 1992) (Groot et al. 1990). Others deal with process simulations and pointed out the energy savings in the downstream recovery of the products from the aqueous broth (Oudshoorn et al. 2009, Qureshi et al. 2005). However, there is only scarce data quantifying the overall energy cost associated to the integration of a liquid-liquid extraction technique. Outram et al. (2016) compared several ISPR techniques by whole integrated simulation, considering downstream but also specific energy demand of each separation technique implementation. In that work, the overall energy demand of the integrated process with oleyl alcohol doubled the one required for conventional distillation scheme. Nevertheless, solvents with different physico-chemical characteristics might lead to different process and regeneration schemes and alter conclusions. Dalle Ave and Adams (2018) performed exhaustive techno-economic comparison of ABE extractive fermentation process based on toxic and nontoxic extractants. The simulations were optimized on the basis of specific features of each extractant, and 2-ethyl-1-

hexanol based-scheme resulted the more profitable option. However, none of these works considered the impact of the physiological response of the microorganism towards the presence of the solvent.

The potential of in situ LLE to improve ABE fermentation has been evaluated in previous lab-scale assays with a vegetable-oil (VO) based solvent and a C12 based Guerbet alcohol (2-butyl-1-octanol, 2B1O) (Chapter 3 and Chapter 4). In that work, partial alleviation of end-product inhibition allowed to treat more concentrated feedstock and to boost butanol obtention. Moreover, the butanol yield increased in the presence of 2B1O, while the volumetric productivity with respect to the control (solvent-free) fermentation was reduced. Here is the first study that considers the solvent-dependent physiological response of the microorganism in the techno-economic evaluation of the whole integrated process.

The objective of this work is to perform solvent-based comparative assessment of ABE extractive fermentation on the basis of the main cost drivers: capital investment, feedstock, total plant energy requirements and wastewater treatment associated cost. Experimental data were available to account for actual fermentation performance parameters and for thermodynamic interactions between phases, resulting in a more realistic view of the integrated process. The techno-economical comparative evaluation is a useful tool to guide research and to identify key challenges to be tackled when revisiting old conventional ABE fermentation.

6.2 Materials and methods

6.2.1 Project assumptions

The plant size selected was set up at 35 ktpa of butanol, which is the typical capacity of a retrofitted mid-sized bioethanol plant (Outram et al 2016). The common assumptions of the simulated scenario are the following:

- Plant production rate: 35 ktpa butanol. It is important to notice that it may imply variations on feed rate or co-products according to specific process parameters of each simulated case (yield structure pattern)

- The experimental performance parameters obtained with each solvent in lab-scale batch extractive fermentations with *Clostridium acetobutylicum ATCC824* were considered.

- Fermentations were carried out in batch or fed-batch mode, depending on assumed total glucose consumption, and were run up to a maximum of 10 g/L of butanol in aqueous phase.

- Regeneration of the solvent occurred concomitantly to the fermentation, to assure renewal capacity up to the end of the run. The enriched ABE fraction issued from regeneration unit was mixed to depleted ABE fraction (aqueous phase in the fermenter) before entering the beer column of the Downstream Section (DS).

- The fermentations are sequenced to assure a continuous feeding to DS operation. The volumetric flowrate entering the DS is function of the final butanol concentration.

- Acetic and butyric acids are assumed to be consumed at the end of the fermentation cycle, then no interaction with subsequent downstream separation was considered. Inhibitors that would be present in aqueous broth of ABE production from lignocellulose (phenolics, furans, and organic acids) were considered to have a negligible influence in the downstream processing.

- The downstream processing route via distillation scheme was based on literature (Mariano et al. 2011). Final product concentration for acetone, butanol ethanol and water were 99.5%, 99.5%, >85% and 99.8%, respectively.

- The vapor-liquid thermodynamics of the DS and regeneration sections were described by default UNIFAC and NRTL extended parameters from Aspen Plus. Experimental values for partition coefficients were used to predict LL equilibria between ABE metabolites and the extractant (Chapter 2)

- For the sake of economic evaluation (discount of minimum selling price), no energy integration was considered, allowing for conservative comparison of maximum energy demand within each configuration case. Nonetheless, pinch analysis was performed with the simulation software using stream information of each scenario, to estimate the potential of energy saving by heat integration.

6.2.2 Process simulations

The energy and mass balances were solved using the process modelling software Aspen Plus, version 7.3 (Aspen Technology, Inc, MA, USA). The different

scenarios are summarised in Table 6.1. *In situ* extractive fermentation with VO and 2B1O were compared to the control case (solvent-free fermentation). Within the solvent-based scenarios, two levels of total glucose consumption were treated. It was reported that a glucose concentration higher than 100 g/L inhibits growth in ABE fermentation (Roffler et al. 1988). Consequently, fed-batch operation was considered in those cases with highest glucose consumption.

Table 6.1. Scenarios considered in this study

Scenario	*ISPR*	*Separation agent*	*Sub-scenarios*	*Glucose consumption (g/L) **
Base Case	No	None	-	55
Sc1	In situ LL extraction	2B1O	a-Batch	90
			b-Fed-Batch	300
Sc2	In situ LL extraction	Pomace Oil	a-Batch	90
			b-Fed-Batch	300

*Glucose consumption in a fermentation cycle (batch/fed-batch)

In this work, experimental results obtained in batch extractive fermentations were combined with steady state simulations of process blocs that were not evaluated experimentally (regeneration loop and downstream section). Therefore, not only the physical effect of end-product inhibition alleviation was considered, but also the impact of different solvents in volumetric productivity and yield structure pattern at the end of the fermentation. It is classically assumed that the ISPR technique has no physiological impact on the bacteria (Outram et al 2016) (Dalle et Adams 2018). Here, experimental data were available to account for these effects resulting in a more realistic view of the integrated process.

The following sections were considered as the basis for comparative techno-economic evaluation: i) fermentation, ii) regeneration of the solvent, iii) downstream. The methodology applied for evaluation of each section is described below.

i) Fermentation

Batch and fed-batch extractive processes were designed to ferment 90 and 300 g/L of glucose respectively. Experimental productivity and mass yield of extractive

batch fermentations with both solvents were considered (Table 6.2). In previous works, 2B1O was proved to modify yield and co-product ratio in ABE extractive fermentations (Chapter 2 and Chapter 3) and to lead to higher culture lag times and degraded productivity (Chapter 3). In a conservative manner, the lowest experimental productivity obtained in bioreactor was considered. It was assumed that these performance parameters are kept during scale-up and within fed-batch operation also.

Table 6.2. Experimental performance parameters (Chapter 2 and 3)

		Control	*VO*	*2B1O*
Yield	Y_B (g/g)	0.18	0.19	0.28
	Y_A (g/g)	0.09	0.11	0.07
	Y_E (g/g)	0.03	0.04	0.05
Productivity	$P_{vol,\ B}$ (g/l/h)	0.20	0.20	0.16
Partition coefficient	K_B		0.62	6.76
	K_A		0.39	1.03
	K_E		0.24	0.61
Selectivity	Sel_B		577.6	644.8

The final total theoretical ABE (B_T, A_T, E_T) concentration is set by the level of glucose consumption (Δ_{Glu}) and yield in each scenario. For butanol, it was calculated as follows:

$$B_T = Y_B \cdot \Delta_{Glu} \quad (6.1)$$

The aqueous butanol concentration must be kept under the toxicity threshold (B_{crit}). This sets the minimum required volumetric ratio of extractant to aqueous phase (V_{ext}/V_{aq}) to remove the produced butanol according to butanol partition coefficient (K_B):

$$V_{ext}/V_{aq} = (B_T/B_{crit} - 1)/K_B \quad (6.2)$$

According to lab-scale experimental results, liquid-liquid thermodynamic equilibria was assumed to be maintained through the fermentation. Thus, aqueous and organic product distribution in the biphasic bioreactor was set based on equilibria assumptions. Water (W) extraction was also considered through the butanol selectivity (Sel_B, Table 6.1) since it might influence the regeneration section. For butanol, it was estimated as follows:

$$B\ aq = (B_T/(1+K_B \cdot V_{ext}/V_{aq}) \quad (6.3)$$

$$B_{org} = K_B \cdot B_{aq} \quad (6.4)$$

Experimental butanol productivities were modified ($P^m_{vol,B}$) considering total cycle time (t_{TC}), which includes fermentation (t_f), transfer to downstream section (t_{DS}) and tank turnover (t_{to}). The latest was fixed to two hours in all cases, whereas t_{DS} varied according to the final butanol concentration. The total bioreactor volume (V_{TOT}) required to achieve the fixed production rate of butanol (m_B) is calculated as follows:

$$P^m_{vol,B} = P_{vol,B} \cdot t_f/(t_f+t_{DS}+t_{to}) \quad (6.5)$$

$$V_{TOT} = m_B/ P^m_{vol,B} \quad (6.6)$$

Each individual bioreactor was assumed to have a total volume of 500 m^3, a height to diameter ratio of 1.5 and a working volume capacity of 75%. When fed-batch is applied, the necessary volume for aqueous feed is integrated in the calculus assuming feedstock concentrated up to 500 g/L. For *in situ* extractions, the volume of the extractant in the bioreactor was set to 25% of the total aqueous volume and was also considered when sizing the tank. Table .6.3 summarizes the data basis of the fermentation section.

Table 6.3. Product distribution, volume of extractant and number of bioreactors

	Units	*Control*	*Sc1-a*	*Sc1-b*	*Sc2-a*	*Sc2-b*
B_T	kg/m^3	10.00	25.35	84.51	17.11	57.04
A_T	kg/m^3	5.00	6.39	21.31	9.83	32.76
E_T	kg/m^3	1.67	4.31	14.35	3.32	11.07
B_{aq}	kg/m^3	10.00	10.00	10.00	10.00	10.00
A_{aq}	kg/m^3	5.00	5.18	9.98	6.79	8.28
E_{aq}	kg/m^3	1.67	3.78	8.58	2.60	3.92
B_{org}	kg/m^3		67.60	67.60	6.20	6.20
A_{org}	kg/m^3		5.34	10.28	2.65	3.23
E_{org}	kg/m^3		2.31	5.24	0.62	0.94
W_{org}	kg/m^3		10.29	10.18	1.05	1.05
V_{ext}/V_{aq}			0.23	1.10	1.15	7.59
$P^m_{vol,B}$	kg/m^3/h	0.182	0.154	0.157	0.189	0.195
V_{TOT}	m^3	24179	28547	28061	23371	22651
N bioreactor		33	49	67	40	54

ii) Regeneration

Upon assumption of liquid equilibria in the biphasic bioreactor, there was no need to enhance mass transfer surface by an external mixer/settler unit, enabling easier phase separation. General proposed scheme for solvents regeneration is presented in Figure 6.1.

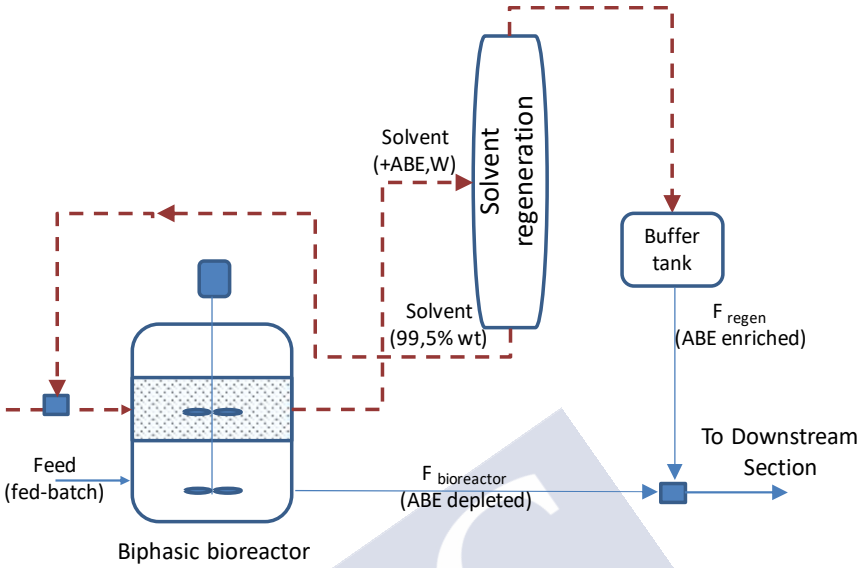

Figure 6.1. Ggeneral regeneration loop (dashed line)

Organic phase composition and total amount of solvent (Table 6.2) were used as an input of the simulation of the regeneration section of each scenario. Only water, acetone, butanol and ethanol were considered in the extractant phase. The final acid concentrations were assumed to be negligible. It was assumed that the regeneration of the extractant occurred concomitantly to the fermentation. Extractants were recovered to a minimum purity of 99.5 % wt before being recycled to the bioreactor. The ABE enriched stream issued from regeneration section is mixed with the ABE depleted stream corresponding to the end of the fermentation cycle, before entering the beer column of downstream section.

Based on preliminary analysis of ternary diagrams (butanol-water-solvent), an homogeneous azeotrope 2B1O-water (32/68% wt) was found at atmospheric pressure and 97.5 ºC. Since the azeotrope converges to pure water composition at 0.2 bar, the regeneration was performed under vacuum, minimizing solvent loses at the top of the regeneration column. Triolein was used as the model compound for vegetable oil in Aspen simulations. A flash unit operation at 250 ºC (which is below to thermal degradation limit in vegetable oil (Dweck and Sampaio, 2004) was enough to regenerate the oil up to the specification (99.5%wt). Figure 6.2 presents the specific regeneration loop configuration for each extractant, with the respective operating conditions

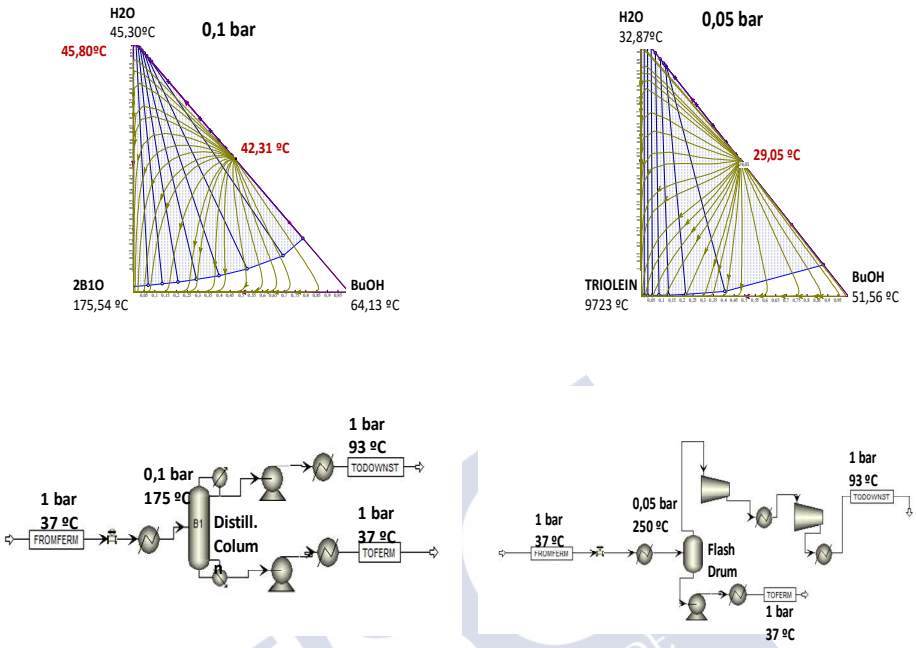

Figure 6.2. Regeneration of solvent simulation: a) 2B1O and b VO

The energy requirements of the regeneration section can be estimated with the elements described above. Energy costs for this section include heating and cooling, electricity for pumping, assuming 100% efficiency.

iii) Downstream section

The aim of the downstream section is to recover acetone, butanol and ethanol from the fermentation and regeneration sections. Butanol production rate set the volumetric flowrate entering the downstream section (Q_{DS}), which is calculated as follows:

$$Q_{DS} = m_B / C_{B,tot} \quad (6.7)$$

The distillation scheme was based upon the work of Mariano et al (2011). The first step is the beer column, used to remove water and solids from the broth. Then, acetone and ethanol are removed sequentially, prior to the heteroazeotropic distillation system involving the purification of both water and butanol, within two distillation columns integrated with a decanter. The simulated flowsheet with the corresponding operating conditions is presented in Figure 6.3.

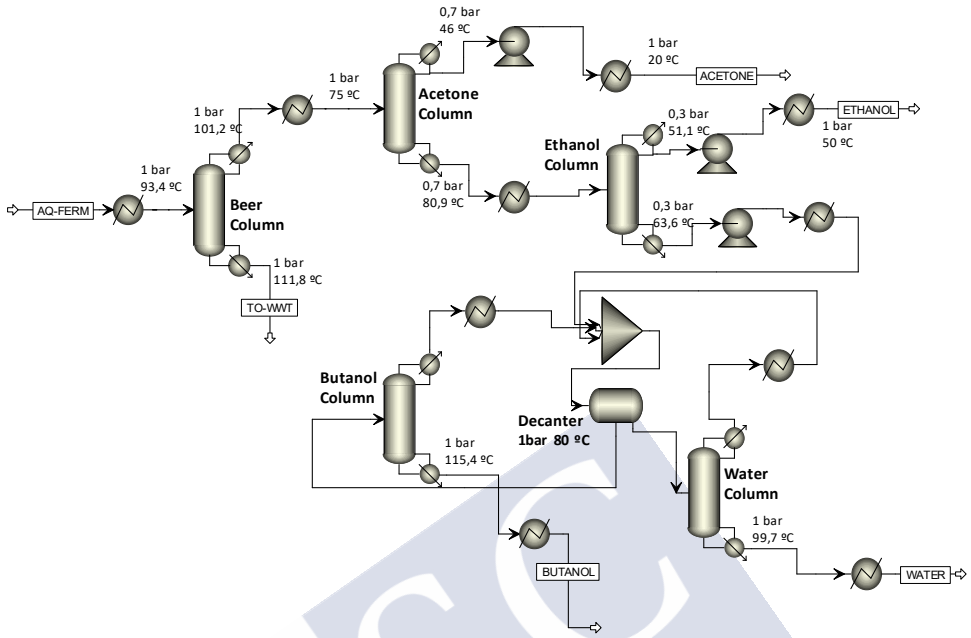

Figure 6.3. Simulation of the Downstream Section

The design/vary feature of Aspen was used to hold design specifications in terms of recovery or purity for specific compounds in either the top or the bottom of each distillation column. These specifications refer to the beer column (maximum 100 mg/kg of ethanol at the bottom), and the recovery product columns, with product concentration (purity%) for acetone, butanol, ethanol and water set to 99.5%, 99.5%, >85% and 99.8% respectively (in respective columns, Figure 6.3). The RadFrac model available in the simulator was used, and full efficiency for columns trays was considered. The number of stages and feed location were fed to the simulator on the basis of previous works or by manual optimization to decrease thermal duties. Comparative energy and investment cost related to downstream purification can be calculated from this scheme.

6.2.3 Economic analysis

Economic analysis was performed based on the results of the simulations.

The fixed capital investment (CAPital EXpenditure, CAPEX) for the different scenarios was estimated with Aspen Process Economic Analyzer (APEA) module. CAPEX included the purchased equipment, equipment setting, and others associated cost (piping, civil engineering or instrumentation).

OPerating Expenditure (OPEX) includes raw materials, utilities, labour, maintenance and other associated costs. Variable operating costs related to heating/cooling requirements or electricity for pumping were based upon the mass and energy balances from the process simulations. Fixed operating costs included items such operating labour, maintenance or plant overhead and were estimated using the APEA module.

Raw material (RM) and wastewater treatment (WWT) were excluded from the total OPEX. The sugar obtention section from raw material (pre-treatment, enzymatic hydrolysis) was not considered in the simulations. Instead, glucose price was assumed to integrate the cost related to this section and to account for the mass yield recovery in sensitivity analysis. Initial base price was fixed at 279 euros/ ton according to spot market (Table 6.4). It was assumed that water obtained at the bottom of the beer column might be sent to WWT, whereas the water obtained from the heteroazeotropic distillation system (about 5% of the total water turnaround) can be directly recycled to the process. The cost of the WWT was set to 4.8 €/t, for a mean BOD of 25 in the stream (Roffler et al 1987)

The initial extractant inventory was considered in fixed working capital, since it was assumed to be regenerated through the successive cycles with minimal loss. Extraction solvent prices considered were 6.2 €/kg (import/export web source) and 2 €/kg (International Olive Council, 2018) for 2B1O and VO, respectively. Variable costs and selling products revenues are presented in Table 6.4. All fermentation products are expected to be valorised. The cost of cryogenic CO_2/H_2 separation was discounted to account for net selling hydrogen price.

The minimum butanol selling price (MBSP) is determined for each simulation case by applying a discounted free cash flow analysis (DFCF). The MBSP is defined as the price of butanol such that the NPV (Net Present Value) of the plant is null over the plant lifetime. The 35 ktpa butanol plant is assumed to operate during 20 year with a discount rate of 8% (weighted average cost of capital) per year. Table 6.4 summarizes the input parameters used for the DFCF analysis. Working capital is assumed to be a fixed percentage of total capex, 5% in the base case, and 7% for the extractive configurations.

Table 6.4. Input for economic analysis

Variable operating cost

Item	*Unit*	*Price*	*Source*
Sugar	€/t	279	USDA, 2019
Steam	€/t	23.5	Nitzsche et al.,2016
Electricity	€/MWh	90.0	Nitzsche et al.,2016
Water	€/t	0.26	Qureshi et al., 2013

Selling prices

Item	*Unit*	*Price*	*Source*
Butanol	€/t	1152	ICIS,2019
Acetone	€/t	675	ICIS,2019
Ethanol	€/t	650	ICIS,2019
H2	€/t	2000	Plan Deploiement Hydrogene (FR), 2018
CO2 separation	€/t CO2	39.7	Air Liquide,2019
Residual biomass	€/t	220	Malmierca et al. 2017

Discounted free cash flow analysis

Production length	20 year
Operating factor	8000 h
Income tax	35%
Depreciation model	Linear
WACC	8%
Working capital	5% / 7% of the total CAPEX

6.3 Results and discussion

6.3.1 Process simulations

The mass and energy balance sheets of the simulations are presented in Table 6.5. They were used as basic inputs for the economics comparative assessment. F_{regen} and $F_{bioreactor}$ refer to both streams entering the separation section (see Figure 6.1). $F_{DSout,\ P}$ corresponds to final P product obtention after downstream recovery (Figure 6.2). The ISPR efficiency is estimated as the ABE products coming from *in situ* separation related to total solvent production. It can be observed that fed-batch operation boosted the efficiency up to 60 and more than 100% with 2B1O and VO respectively under the assumptions of this work.

Table 6.5. Mass and energy balances. Simulation results

Variable	*Units*	*Control*	*Sc1-a*	*Sc1-b*	*Sc2-a*	*Sc2-b*
F_{regen},B	kg/h		2669.2	3886.2	1753.2	3497.2
F_{regen},A	kg/h		210.8	591.1	763.8	1852.6
F_{regen},E	kg/h		91.1	301.0	179.1	537.8
F_{regen},W	kg/h		406.1	585.5	308.5	611.1
$F_{bioreactor}$,B	kg/h	4408.2	1738.7	521.6	2576.2	772.9
$F_{bioreactor}$,A	kg/h	2204.1	901.0	520.6	1749.4	639.6
$F_{bioreactor}$,E	kg/h	734.7	657.6	447.7	670.8	303.3
$F_{bioreactor}$,W	kg/h	433469.4	170573.0	50671.1	252621.0	75569.5
F_{DSout},B	kg/h	4360.6	4386.1	4392.4	4292.0	4249.6
F_{DSout},A	kg/h	1887.8	1056.2	1111.6	2296.9	2487.3
F_{DSout},E	kg/h	711.9	736.3	732.4	831.3	823.9
F_{DSout},W	kg/h	433330.6	170838.4	51116.0	252769.2	76019.6

Variable	*Units*	*Control*	*Sc1-a*	*Sc1-b*	*Sc2-a*	*Sc2-b*
Energy consumption (regeneration)	MJ/kg ABE $_{regen}$		15.84	14.67	2.83	2.70
	MJ/kg B $_{regen}$		17.63	18.04	4.33	4.52
Energy consumption (DownStream)	MJ/kg ABE $_{prod}$	34.6	19.1	11.4	22.5	11.2
	MJ/kg B $_{prod}$	55.2	26.8	16.2	36.9	16.0
ISPR efficiency	ABE $_{ISPR}$ / ABE $_{TOT}$		47%	76%	36%	80%
	B $_{ISPR}$/ B $_{TOT}$		61%	88%	42%	85%

Downstream section

Figure 6.4 shows the simulated decrease of energy in the downstream section per unit of product as a function of aqueous butanol concentration in the inlet beer stream. In Figure 6.4, continuous lines were plotted considering typical ABE ratio corresponding to base case (circle). Data obtained for different scenario of this work were plotted over base lines and differences rely on ABE product ratio variations between cases. The highest energy sensitivity is found in the more diluted region (<10 g/L), which corresponds to typical broth concentration in ABE conventional fermentations. Therefore, small increments in butanol concentration entering the beer columns may greatly reduce the energy consumption. Extractive batch operations that were designed to enhance glucose consumption of 60% in relation to the conventional batch would lead to energy demand reductions by 51% and 33% with 2B1O (Sc1-a) and VO (Sc1-b), respectively (Fig 6.4). In fed-batch operation, the energy efficiency of the distillation train was significantly improved with the VO solvent (Sc2-b), resulting in a 56.7% saving over the batch extractive (Sc2-a). Energy requirement decreased by 39.6% with 2B1O fed-batch mode (Sc1-b) over corresponding batch (Sc1-a). As observed in Figure 6.4, the energy consumption asymptotically approached 10 MJ/kg ABE when inlet butanol concentration increases, and no clear decrease is appreciated for values higher than 50-60 g/L. This agrees with data presented in (Mariano et al. 2011). Consequently, fed-batch simulations presented modest improvements in terms of energy reduction in DS despite reaching three times higher final butanol concentrations compared to the corresponding batch operations.

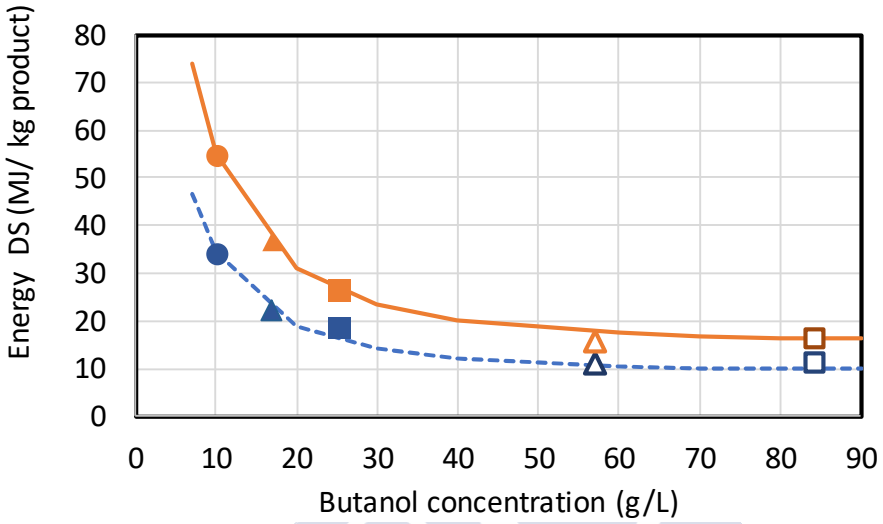

Figure 6.4. Maximum energy requirement for the downstream section vs inlet aqueous concentration. Base case (circle), Vegetable Oil (batch – full triangle-, fed-batch – void triangle-), 2B1O (batch – full square-, fed-batch – void square-). Base lines (continuous) considered Base Case ABE product ratio (MJ/kg B, orange, MJ/kg ABE, blue)

Integrated process energy requirement

The energy requirement for the implementation of the in-situ product recovery technique must be included in total energy balance. Figure 6.5 shows the maximal downstream and ISPR contribution to total energy demand. For comparison purposes, both energy aspects are expressed in equivalent units (MJ/kg ABE produced). The conventional batch case presented the highest total energy demand (35 MJ/kg ABE), corresponding to the DS requirements, whereas the fed-batch extractive operation with vegetable oil reduced by more than a half the total consumption (13.4 MJ/kg ABE). This sharp reduction is driven by the low energy cost of VO regeneration compared to 2B1O. In fact, despite needing 10-times more VO extractant due to its low capacity (K = 0.6), about 3 MJ per kg of ABE regenerated were required for VO-based extractive fermentations, instead of 15-16 MJ/kg ABE when using 2B1O (Table 6.5). From the energy point of view, the best option would be to work with VO extractive fed-batch operation.

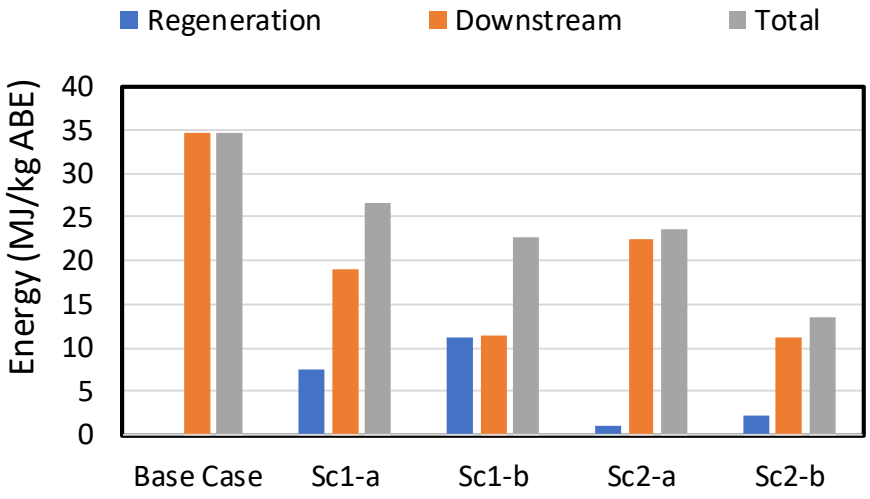

Figure 6.5. Maximal energy requirements (Downstream, Regeneration and Total) for batch (Sc1-a: 2B1O, Sc2-a: VO) and fed-batch (Sc1-b: 2B1O, Sc2-b: VO) extractive fermentations

Comparison with previous studies is difficult since each work might rely on very different assumptions. Outram et al (2016) compared different ISPR techniques based on energy demands (similar approach to this work) and found that liquid-liquid extraction with oleyl alcohol presented the highest requirement for regeneration (100 MJ/kg ABE, which is quite higher than values obtained in this work). In their simulations, they included external equipment (mixer/settler) that was not considered here, since liquid equilibria was based on experimental data. Moreover, oleyl alcohol presents a lower butanol partition coefficient (K = 3) than 2B1O, which means that more solvent needs to be regenerated to reach the same recovery degree.

Heat integration

Figure 6.4 presents the maximum energy requirements for a given inlet butanol concentration. Energy savings on heating and cooling can be made by heat integration. Detailed analysis on possible heat integration in downstream butanol recovery was published in (Gonzalez-Bravo et al. 2016). Here, pinch analysis was performed for both downstream and regeneration section by using the Energy Analysis module of the simulation software to estimate the potential of energy saving by heat integration

Concerning downstream section, heat integration would allow to save up to 40% energy in the base case scenario, and 9% for fed-batch configuration, with higher butanol concentration in the beer stream. Nonetheless, the relative ordering for energy downstream requirements of studied cases is not altered. The data obtained agree with previous work. Mariano et al. (2011) stated that heat integration contributed with an energy reduction of 4.6 MJ/kg butanol for inlet alcohol concentration of 17 g/L, while reduction of 4.3 MJ/kg was estimated in Sc2a (same inlet butanol concentration).

In regeneration section, composite curves analysis determined 30% savings in heating/cooling energy for Sc1 a,b (2B1O scenario) while up to 85% of energy reduction in VO-based configurations. Finally, total energy reduction (downstream and regeneration) with heat integration varying between 40% for Base case, Sc1 a,b and 20% for Sc2 a,b. Nonetheless, for comparison purposes the relative ordering in terms of total energy requirement is maintained between the five scenarios.

Further work would be required to determine the optimal exchange network to achieve the minimal energy requirement and to include the associated capital expenditures in the integral economic analysis.

6.3.2 Economic evaluation

Cash flow analysis

The annual cash flow corresponding to capital and operational costs, and products sales is presented in Figure 6.5. Butanol revenues are similar in all configurations as expected since they share a common butanol production rate. Variations in side products revenues are due to the differences on co-products ratio in extractive fermentations with 2B1O. As can be deduced from the Figure 6.5, with input assumption, net margin is negative and production cost is dominated by raw materials (RM) in all studied configurations. For the control case, RM represents almost 60% of total production cost (73% of operating cost), and annual RM cost (55 M€) is close to total products revenue (58M€). This is in agreement with (Dalle Ave et al. 2018), where annual cost for feedstock and total side product revenue were 58 and 52 M€ respectively in the base case.. In (Outram et al. 2016) RM accounted for 72% of total OPEX in solvent-free configurations. Other work estimated lesser impact of the feedstock cost, as in (Qureshi et al. 2013) or (Malmierca et al. 2017) where RM accounted for 26% (wheat straw) or 33% (corn stover) of the total OPEX. Although being the most elevated expenditure in all scenarios, raw materials cash flow is 34% less important in extractive fermentation, particularly with 2B1O (Sc1-a, Sc1-b), clearly due to the butanol yield improvement observed with this solvent.

In regard to annual cost, the second largest contribution is the wastewater treatment when no ISPR technique is applied. The obtention of more concentrated aqueous broth at the end of the fermentation-ISPR section has direct consequences in WWT cost (Fig 6.6). It accounts for 17% in the control case while not more than 10.1% and 11.5% for the batch extractive operations (Sc1-a and Sc2-a respectively). In the case of the fed-batch operations WWT cost contributed even to a lesser extent (3.1 and 3.7% of total production cost for Sc1-b and Sc2-b respectively). In these cases, total water turnaround is reduced in more than 80% over the conventional batch system.

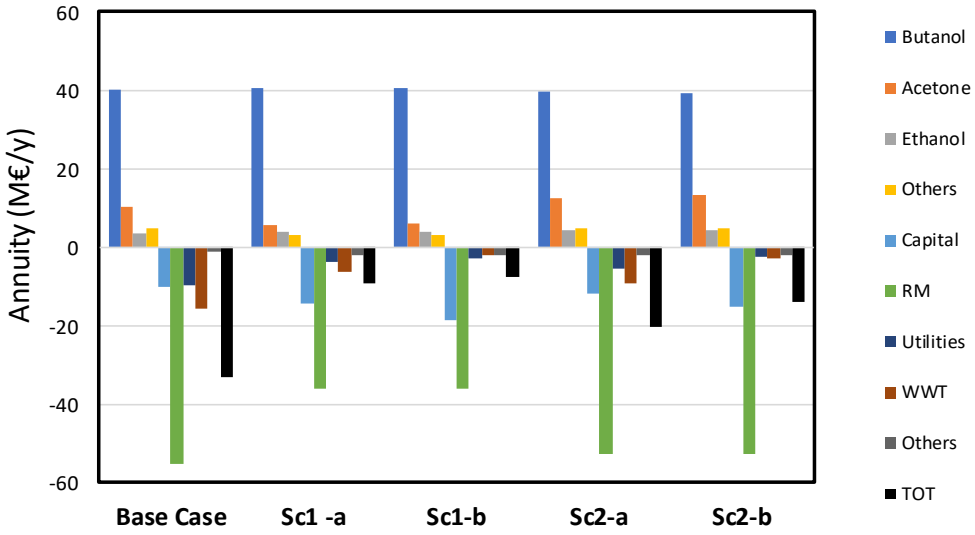

Figure 6.6. Annual cash flows for all scenarios

Differences between the relative contribution to the total annual production cost can be better appreciated if the raw material cost is excluded (Figure 6.7). For the comparative analysis, the WWT cost has been also separated from the remaining OPEX. As deduced from Figure 6.7, both WWT and OPEX are correlated with the product concentration reached before entering the downstream section. However, the product concentration enhancement is more beneficious in terms of WWT savings, particularly in fed-batch operations. As a result, the WWT cost relative contribution decreased from more than 40% in the base case (Figure 6.7) to 12.5 and 7.5% in fed-batch extractive fermentations with 2B1O and VO, respectively. In these cases, WWT represented not more than 30 and 40% respectively of the total operating cost, while it accounted for more than a half in the rest of the scenarios. In spite of the topped energy economy in DS (Figure 6.4) and the additional cost required to implement the ISPR (FIgure 6.5), the effort of

concentrating the in/out streams of the ABE process had an overall benefit in terms of reduction of WWT cost.

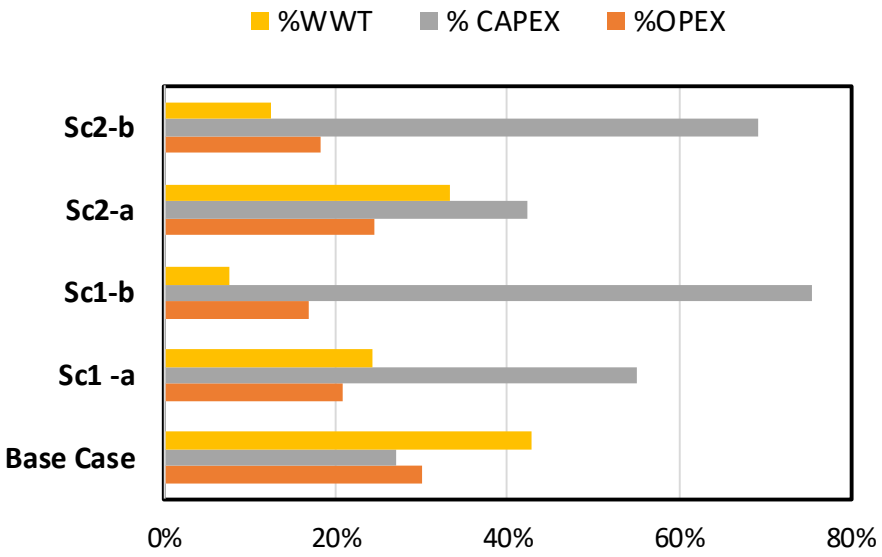

Figure 6.7. Relative contribution for CAPEX, WWT and OPEX (excluding RM and WWT)

Capital investment is a critical economic aspect presenting important differences between the simulated cases (Figure 6.7). In fact, CAPEX is associated with fermentation section (contribution of more than 85% for all scenarios). The additional capital investment required to implement the liquid-extraction varied from 16 M€ in the VO-based batch case to 81 M€ with 2B1O, in fed-batch configuration. Outram et al- (2016) reported an extra capital of 44.6 M€ for the ABE extractive process with Oleyl Alcohol over solvent-free case. The huge volumetric capacity required, estimated from process productivity, is responsible for this additional cost and for its significant variability among the studied cases. Conventional ABE fermentation has intrinsic low productivity, mainly hindered by strong end-product inhibition. The use of ISPR techniques aims at alleviating this toxicity and enhance total solvent production by extending fermentation time. Theoretical enhancement of intrinsic kinetics (volumetric productivity) has been considered as an input for techno-economic evaluations of ABE-ISPR integrated process (Dalle Ave et al. 2018, Mariano et al. 2011) Validated models quantifying the influence of end-product inhibition on growth have been applied to predict the kinetics under product toxicity alleviation. However, eventual negative influence of the solvents, specially the most capacitive ones, that might counterbalance the benefits of removing toxic products from the broth, had not been considered. In

this work, experimental performance with the two specific solvent was considered, thus accounting for real volumetric productivities. 2B1O presented biphasic toxicity and reduction of 20% of productivity over control case. While, vegetable oil resulted in alcohol overproduction, without significant productivity enhancement. Variations of total bioreactor volume between scenarios must account for these variations of productivity. In addition, *in situ* extractive fermentation avoids the use of an external separation unit, but in detriment of a percentage of bioreactor volume (set to 25% in this work) that must be dedicated to the extractant. Finally, the additional cost of fed-batch mode is also integrated in terms of an additional void initial volume in each bioreactor. As a result, different number of bioreactors reflected important differences in CAPEX. Particularly, fed-batch operation is clearly impacted reaching a CAPEX contribution of 78% (Figure 6.7) for the SC1-b scenario.

Figure 6.8 presents the evolution of the number of bioreactors as a function of the process volumetric productivity, accounting for experimental observed productivity degradation in the presence of 2B1O. It is interesting to note that volumetric productivity might be significantly increased to perceive a clear decrease in the number of the bioreactors required and thus in CAPEX. The application of alternative solutions to enhance productivity, for instance, bioreactor intensification by cell density increase or biofilm application might be necessary to significantly reduce the bioreactor volume and to generate more important investment savings. For instance, volumetric productivity was increased by more than 10-times when biofilm technology was applied to ABE fermentation in continuous mode (Qureshi et al. 2004). Furthermore, synergies between cell immobilization and solvent-based ISPR technique have already been announced in literature for ethanolic fermentation (Aires-Barros et al. 1986, Kapucu and Mehmetoglu, 1997). In these works, the authors proved that immobilization conferred an additional protection against toxic or inhibitory solvents. This complementary approach might be explored in ABE extractive fermentation.

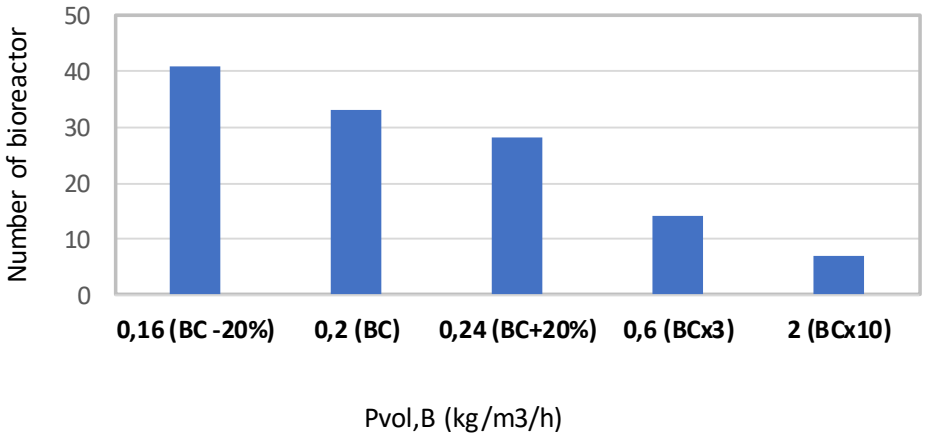

Figure 6.8. Number of bioreactor (1000 m^3) as a function of Base Case (BC) butanol volumetric productivity (P vol, B).

Minimum Butanol Selling Price (MBSP) and sensitivity analysis

The profitability values of the different scenarios are compared through the Minimum Butanol Selling Price in Figure 6.9. The pure distillation base case resulted in the highest MBSP value of 2.18 €/kg of biobutanol produced. This value agrees with previous reported economic evaluation of fermentative butanol production. Qureshi et al. (2013) set the MBSP to 2.11€/kg for a grass rooted nonintegrated plant of the same butanol capacity, while Dalle Ave et al. (2018) obtained 2.66 €/kg for a butanol capacity of 85 Mtpa. Here, all extractive configurations performed better than the base case, with minimum MBSP corresponding to 2B1O-based extractive fermentations (1,54 and 1.53 € for Sc1-a and Sc1-b respectively)

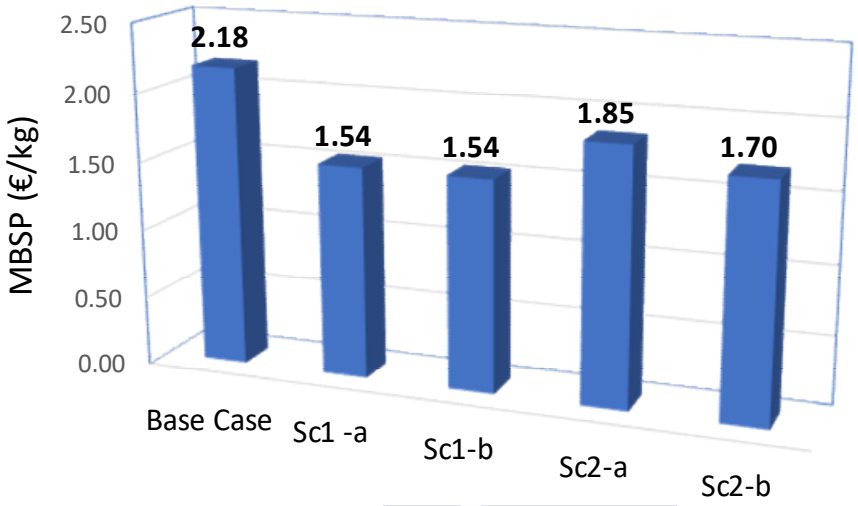

Figure 6.9. Minimum Butanol Selling Price for all scenarios

The variations between cases responded to relative savings and extra investments between cases and over conventional case in (Figure 6.10). 2B1O-based batch fermentation resulted in 20% lower MBSP compared to VO-batch (Figure 6.9). From Figure 6.10, it can be deduced that enhanced butanol yield with 2B1O compensated higher CAPEX requirements (due to lower productivity). Fed-batch operation performed better that the corresponding batch in the case of VO extractant. Despite higher demand in terms of CAPEX (due to fed-batch regime), the higher product concentration resulted to counterbalance the investment cost by reducing energy for downstream and WWT related costs.

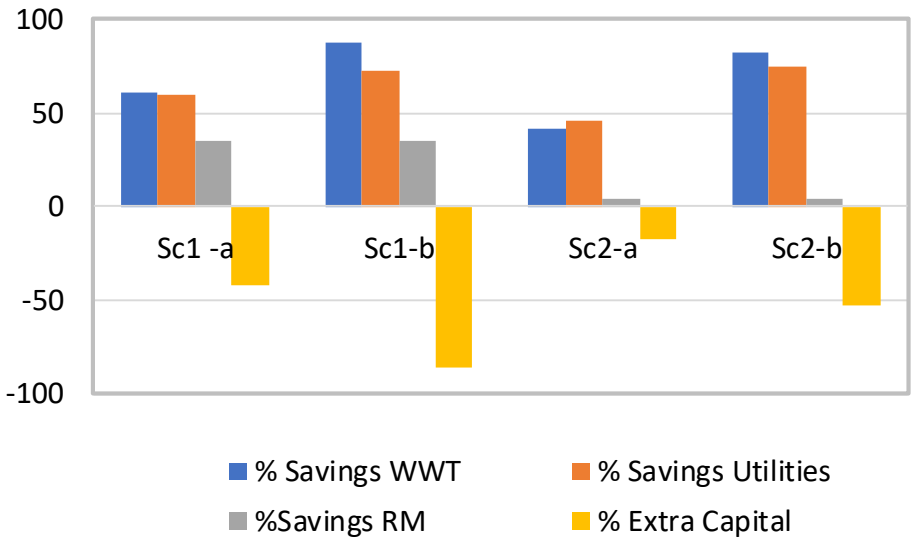

Figure 6.10. Relative savings and Extra Capital over Base Case

The economic profitability of the project is guaranteed only if the MBSP is equal or lower than the butanol price in the market. Under the economic assumptions considered in these work, production and investment cost might be still reduced in all scenario to match the market price of butanol (1152 €/ton). Nevertheless, the MBSP obtained for integrated configurations, especially for Sc1-a,b and Sc2-b, are closer to the fluctuating price of butanol (ICIS, 2019). Moreover, biobased products might benefit from fiscal advantages compared to fossil-based equivalents. Considering these last considerations, the intensified scheme seems much more promising.

The relative order of performance between the different simulated scenario is not maintained through a sensitivity analysis on main cost parameter, the sugar price, as can be observed in Figure 6.11. Because of enhanced butanol yield, the differences between solvents increase in favor of 2B1O as the cost of the feedstock increases. On the contrary, integrated process with Vegetable Oil, specially fed-batch operation, could be a more interesting option with the use of cheaper substrate. In Figure 6.11, rentability of the fed-base VO-based configuration (Sc2-b) becomes higher than that of 2B1O cases (Sc1 a,b) if the cost of sugar is lower that approximatively 200 euros/ton of sugar. This value also corresponds to the maximum sugar cost allowing MBSP to approach “rentability zone”, which included butanol market price fluctuation from the beginning of 2017 (ICIS 2019). This feedstock price considered here comprises the complete raw material preparation section (pretreatment, hydrolysis…). In Sofia Laure et al. (2014), the authors

performed a techno economical assessment of an lignocellulosic Organosolv based biorefinery and estimated that glucose production cost might reach 220 euros/t glucose, which is close the critical required value in Figure 6.11.

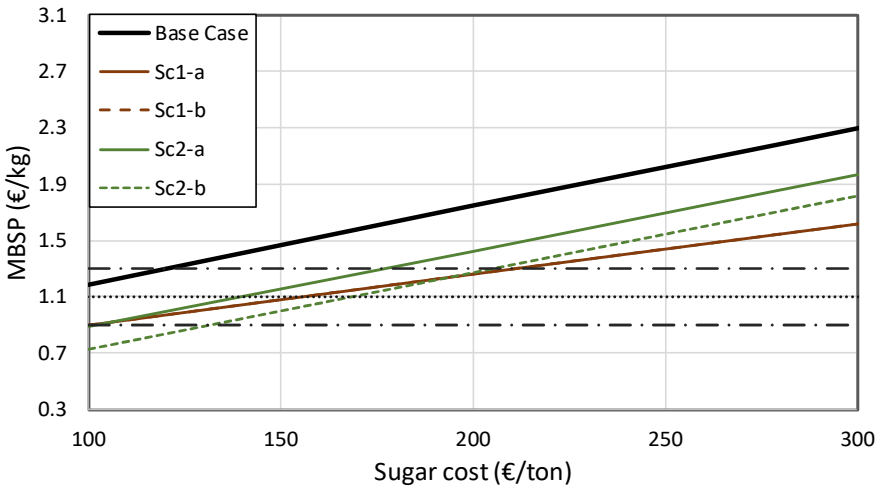

Figure 6.11.en Sensitivity analysis on sugar cost (Horizontal lines correspond to window market price since 2017)

Less sensitivity is appreciated on co-product selling price variation, and relative ordering of scenario is not altered (Figure 6.12) considering acetone market price fluctuation since the beginning of 2017 (ICIS, 2019) It can be concluded from simulated data that acetone price should over pass 950 €/ton in order to let fed-batch process with VO solvent became the most interesting option, thanks to co-product valorization.

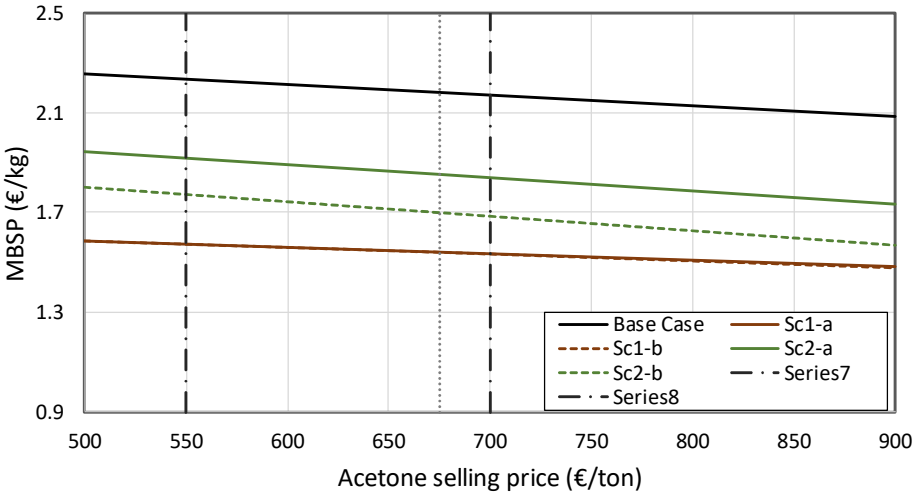

Figure 6.12. Sensitivity analysis on acetone selling price (Horizontal lines correspond to window market price since 2017)

6.4 Conclusions

Economic comparative assessment of an ABE extractive fermentation plant producing 35 ktpa of butanol per year was performed based on the extractant used (2-butyl-1-octanol -2B1O- and Vegetable Oil -VO-) and the operating mode (batch and fed-batch). The simulations were fed by experimental data, allowing to consider the real performance (productivity and yield). All integrated configurations decreased the production cost over the conventional batch scheme, and the highest profit increase was found with 2B1O in fed-batch mode, lowering by 29% the minimum butanol selling price over the base case. In this configuration, the economy was driven by 80% reduction in Waste Water Treatment requirements and 34% savings in raw materials.

Although none of the simulated scenario was profitable under the assumptions of this work, the sensitivity analysis indicated that reducing the cost of feedstock (to 200 euros/t sugar) the integrated process could be competitive within the chemical industry.

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7. Integration of ABE fermentation in a 2G-based bio-refinery

ABE fermentation has a high potential for industrial application in lignocellulose-based biorefinery. In a first part of this work, the hydrolyzed cellulose from organosolv pre-treated beech wood was fermented by *Clostridium beijerinckii CECT 508* and compared with synthetic P2 medium in conventional and 2-butyl-1-octanol based extractive fermentation processes. ABE overproduction of 40% was observed with cellulose hydrolysate compared to conventional control fermentation and was increased by 10% with extractive fermentation. However, lag time in fermentations with hydrolysate was doubled, probably due to the presence of remaining inhibitors from the organosolv pretreatment. In a second part, a laccase-detoxification step was applied prior to fermentation, within two possible industrial scenarios: with and without solid-liquid operation after enzymatic hydrolysis. Solventogenesis was fully effective in all configurations, but the benefits of the laccase enzymatic treatment were revealed only when the remaining lignin solids were present in the medium, resulting in a 25% improvement in ABE production. Regarding to further scale up of the process, the direct fermentation after enzymatic hydrolysis would alleviate investment cost and simplify the process scheme.

Part of this chapter has been redrafted after Gonzalez-Peñas H., Lu-Chau T.A., Botana, N., Moreira M.T., Lema J.M., Eibes, G. 2018. Organosolv pretreated beech wood as a substrate for acetone butanol ethanol extractive fermentation. Holzforschung, 37: 577-584.

OUTLINE

7.1Introduction

As stated in previous chapters, current industrial production is highly dependent on fossil resources, which provide 80% of the world's energy output. However, global energy demand is growing steadily, while crude oil reserves are finite. This led to renewed interest in the conversion of biomass into biofuels and biochemical intermediates. The progressive development of biorefineries is in focus of the mid-term strategies with this regard. The biorefinery concept was first defined by the National Renewable Energies Laboratory (NREL, USA) as “a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass”, and can be classified according to the raw material input and the type of products produced. Second generation biorefineries are those that use lignocellulosic materials, including agricultural residues (corn stover, wheat straw, sugarcane straw, bagasse, etc.), forest residues (woody biomass), municipal solid waste or energy crops planted in non-productive areas, aiming at the production of biofuels and biochemicals (Valdivia et al. 2016).

Even if renewable butanol obtention by the ABE (acetone, butanol and ethanol) fermentation is a promising alternative for advanced biofuel production, the three main performance variables of the process (yield, product titer, and productivity) should be increased for the sake of an economic commercial production (Green 2011; Dürre 2011). Substrate cost amounts up to 60% of the overall cost in the ABE fermentation process (Ranjan and Moholkar 2012). From the economic perspective, it is essential to identify abundant and inexpensive substrates that can be fermented by Clostridium species. Therefore, attention has recently been paid to lignocellulosic biomass, particularly residues from agriculture and forestry, to provide a less expensive carbon source for fermentation processes (Zhu et al. 2015).

The effective conversion is another prerequisite for efficient ABE production (Kumar and Gayen 2011). Native cellulose is resistant to enzymatic attack as it is naturally protected by the surrounding matrix consisting of lignin and hemicellulose (Pan et al. 2005). Therefore, pre-treatment prior to the enzymatic cellulose hydrolysis stage is necessary (Taherzadeh and Karimi 2008). The classical dilute sulfuric acid pretreatment is not suitable for ABE production from lignocelluloses without an additional detoxification process (Ezeji et al. 2007). Moradi et al. (2013) applied alkaline and phosphoric acid pretreatments for ABE production from rice straw. Organosolv processes enhanced the effectivity of pretreatment (Hamiri et al. 2014), while during this process a part of the lignocellulosic biomass is dissolved in an organic phase, in the presence of HCl or H_2SO_4 as catalysts leading to breakdown of the lignin and hemicelluloses moiety of wood. After treatment, the cellulose containing fibers are recovered by filtration. Lignin is then precipitated from the solution, and in this way the lignocellulosic material is separated in its

three main constituents. Organosolv pretreatment improves the enzymatic hydrolysis as manifested by the higher yield of fermentable sugars and low energy consumption (Pan et al. 2005). Pine, elm, reed, or beech wood have been subjected to organosolv pretreatment prior to ABE fermentation, with maximum ABE titers in the range of 11.6-15.1 g L-1 (Amiri and Karimi ,2015; Zhu et al. 2015; Tippkötter et al. 2014; Roth and Tippkötter 2016).

Even if the organosolv pretreatment allows extensive delignification of the wood, the residual lignin in the remaining pulp or the presence of released inhibitors during the pretreatment process may hinder the subsequent steps of the process (enzymatic hydrolysis and/or fermentation). Among different delignification and detoxification methods that have been object of investigation, the use of laccases has been considered as a powerful and environmentally friendly tool allowing to boost conversion of pretreated lignocellulosic materials (Fillat et al., 2017). In fact, laccases are copper-containing blue oxidases that catalyze the oxidation of phenolic units in lignin. Then, by generating unstable phenoxy radicals from free phenols, polymerization into less toxic high-molecular-mass products is induced. This way, the reduction of toxic effects of phenolic compounds can be achieved (Fillat et al. 2017, Roth and Spiess, 2015. García-Torreiro et al. (2017) applied laccase detoxification prior to enzymatic hydrolysis of pulp organosolv, but no enhanced sugar yield was observed. This agrees with Jurado et al. (2009), that optimized laccase detoxification step on steam-exploded wheat straw and observed slightly decreased glucose recovery after enzymatic hydrolysis. Nevertheless, in that work, improved fermentability (more than two times higher) was observed for yeast ethanol production after laccase detoxification. The use of laccase on a pre-hydrolysate serving as a substrate for ABE fermentation led to doubled butanol production (from 1.54 to 4.17 g/L) compared to non-treated pre-hydrolysate (Allard-Massicotte et al. 2017), However, in that case, phenolic compounds concentration was well above the inhibition limit reported by Bellido et al. (2018). As far as we know, no information is available for detoxification prior to ABE fermentation in organosolv hydrolysate-based substrate.

Besides yield, the low titer typical of batch ABE fermentation is related to the strong product inhibition of solventogenic Clostridia and needs to be improved to let the process economically feasible. In Chapter 6, it was concluded that integrated extractive process with 2 -butyl-1-octanol (2B1O) might be rentable, according to the butanol market price fluctuation, if the sugar price approached 200 euros/ton. In Sofia Laure et al. (2014), the authors performed a techno economical assessment of a lignocellulosic Organosolv based biorefinery and estimated that glucose production cost might reach 220 euros/t glucose, which is close the critical required value.

In the present work, the hydrolysate of European beech wood (*Fagus sylvatica* L.) was evaluated as a potential substrate for 2G-based ABE fermentation. Even if solventogenic Clostridium can ferment C6 and C5 based sugar, it will preferentially consume glucose due to transcriptional repression of xylose utilization genes (Jones and Woods 1986). The strain here used was *Clostridium beijerinckii CECT 508* (NCIMB 8052) instead of *Clostridium acetobutylicum ATCC824,* used previously in this thesis. Indeed, the former has been reported to boost up to 60% total sugar consumption and butanol production in mixed sugar (xylose and glucose) based substrates (Xin et al. 2014).

The work plan was structured as depicted in Figure 7.1. In a first step (STEP 1) cellulose hydrolysate (HC) based fermentation was compared to synthetic P2 medium. An in situ extractive fermentation system with 2B1O as the extraction agent was applied to mitigate the end-product inhibition and to improve the overall ABE fermentation performance in both configurations. The second part of the work (STEP 2) dealt with the implementation of a detoxification step prior to ABE fermentation with hydrolysate. Two possible industrial scenarios were evaluated: with and without solid-liquid separation unit after enzymatic hydrolysis, EH (centrifugation to remove the remaining solid in the hydrolysate). Previously, optimization of the operating conditions of the base case was achieved to boost glucose consumption and attain higher solvent titer.

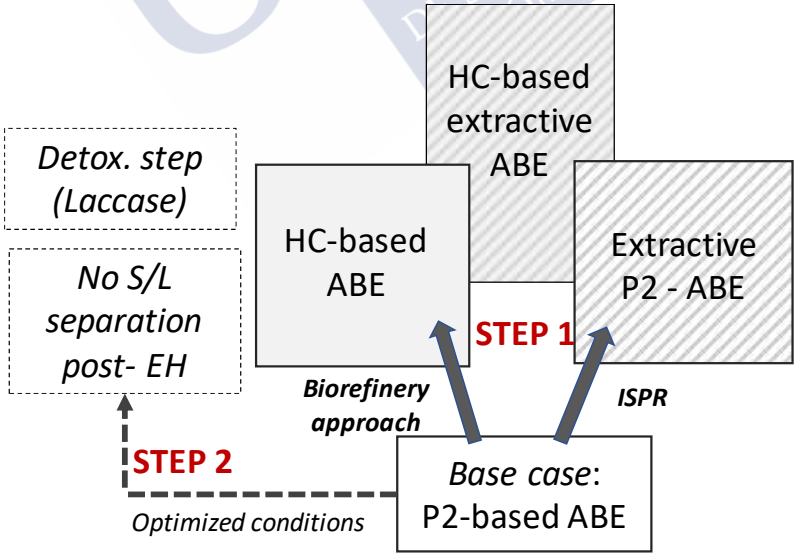

Figure 7.1. Work plan structure of Chaper 7

7.2 Materials and methods

7.2.1 Microorganism and culture media

Clostridium beijerinckii (CECT 508) was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). The strain was activated in a reactivation medium (ReM) in 20 mL flasks, and then reinoculated in 50 mL flasks with 35 mL of ReM. These cultures were stored at 4 °C and used as working cell bank and master cell bank. For preparing the inoculum of the different experiments, 2 mL of the working cell bank were grown in 18 mL of P2 medium for 96 h. The culture conditions were 37 °C, initial pH 6.8, under anaerobic conditions. Prior to inoculation, the abiotic suspensions were purged with N_2 to ensure anoxic conditions at the beginning of the culture. The composition of the RM ATCC 2107 (in g L^{-1}) was: peptone 10, beef extract 10, yeast extract 3, glucose 5, NaCl 5, starch 1, L-cysteine 0.5, and sodium acetate 3. Additionally, 4 mL/L of resazurin were added to monitor anaerobic conditions in the cultures. The composition of the P2 culture medium (in g L^{-1}) was as follows: $FeSO_4.7\ H_2O$ 0.01, $MgSO_4.H_2O$ 0.2, $MnSO_4.H_2O$ 0.01, KH_2PO_4 0.5, K_2HPO_4 0.5, CH_3COONH_4 2.2, NaCl (0.01), p-aminobenzoic acid 0.1, yeast extract 1, resazurin 0.25, and glucose 60. The organosolv cellulose fraction was supplied by Fraunhofer Center for Chemical-Biotechnological Processes (CBP, Leuna, Germany). Its composition was found to be: Klason lignin 14.6%, celullose 74.5%, hemicelluloses 8.6%, and ashes 2.3%.

7.2.2 Enzymatic hydrolysis

The method described by López-Abelairas et al. (2013) with some modifications was applied. Cellulase (50 FPU g^{-1}) and β-glucosidase (250 IU g^{-1}) were supplemented by the addition of the enzyme cocktails Celluclast 1.5 L and NS50010 (both from Novozymes), respectively. Details of hydrolysis: 1 L volume flasks containing 25 g of organosolv cellulose, 0.1 M citrate buffer 250 mL, pH 4.8, solid load of 10% (w/v) for 100 h, orbital shaker, 50°C and 150 rpm. After hydrolysis, half of the solution was centrifuged at 4500 rpm for 10 min. The supernatant and the remaining (not centrifuged) broth were stored at 4°C until use. Finally, reducing sugars and glucose concentration of the supernatant were determined by the DNS (3,5-dinitrosalicilic acid) method (López-Abelairas et al. 2013) and an enzymatic kit (GOD-POD, Spinreact, Spain), respectively.

7.2.3 Laccase detoxification treatment

Detoxification treatment was performed using laccase from *Trametes versicolor* supplied by Sigma Aldrich (lot result 1.07 U/mg). Laccase activity was determined

by measuring the oxidation of 5 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 100 mM sodium acetate buffer, pH 5 (ε_{436}=29.3 mM/cm) (Muñoz et al. 1997). Enzymatic activity was expressed as international units (U), defined as the amount of enzyme that catalyzes 1 µmol of substrate per min.

Preliminary assays were performed to optimize the conditions (temperature, dosage and time of incubation) of the detoxification treatments. These assays were performed on the supernatant of the hydrolysate, 0.1 M citrate buffer (pH 5.5). Two dosage levels (1000 and 5000 U/L) and two temperatures (30 and 50 °C) were tested. The reaction medium was incubated in a 20 mL flask with a magnetic stirrer and for 24 h. Periodical samples were taken to determine enzyme activity and total phenol concentration. After preliminary assays, subsequent enzymatic treatments were carried out using 4000 U/L of laccase and were performed at 30 °C in a 250 mL sealed-flask with magnetic stirring.

7.2.4 Control fermentation

In the first part of this work (STEP 1), duplicate control fermentations with *C. beijerinckii CECT508* were carried out to characterize the process in synthetic P2 medium in terms of pH, substrate and metabolites profiles. First, 250 mL sealed-bottles were filled with 100 mL of fermentation medium and inoculated with 10% volume of cells growing at maximum growth rate (OD600 1.8-2). The system was previously purged with N2 for 30 min to maintain oxygen-free conditions from the beginning of fermentation (37 °C and pH 6, no stirring). Thereafter, pH varied freely during the fermentation. The entire system was autoclaved at 110 °C for 30 min prior to inoculation. Initial glucose was fixed at 60 g/L and samples were taken periodically from the fermentation medium. In the second part of the work (STEP 2), the control fermentations were carried out in triplicates and using 100 mL serum bottles, with 30 mL of fermentation medium. The temperature was set as an optimization variable (30, 34 or 37 °C). The rest of the protocol was unchanged.

7.2.5 Organosolv hydrolysate-based fermentation

In these experiments, the carbon source was replaced by organosolv hydrolysate, which provided an initial glucose concentration of 60 g/L. The rest of the protocol was identical to that described below for control fermentation.

7.2.6 Extractive fermentations

In the first part of the work (STEP 1), extractive experiments were carried out in 250 mL sealed serum flasks filled with 100 mL of culture medium, following the experimental configuration and methodology described in Chapter 3.

7.2.7 Analytical methods

Gas chromatography (GC) was done using an HP6890 instrument (FID), with N_2 as the carrier gas. Agilent DAB-WAX column (Ref. Nº 122-7032), 30 m long and 0.25 mm in diameter, was used. Temperature program: 70 °C for 3 min → 200 °C (60 °C min^{-1}). An individual calibration curve was generated for each compound in the aqueous phase. In the case of extractive fermentation, the metabolites in the organic samples were quantified by using two dilution agents: a) hexane, which helps determine butanol and butyric acid, and b) butanol for quantification of E and A. Acetic acid was not quantified in the organic phase because its partition coefficient in the extracting agent was too low. In both cases, hexane and butanol were combined in a ratio of 2:1 (v/v) with the organic aliquot being analyzed. The objective was to ensure a single homogeneous sample at room temperature prior to GC analysis.

Glucose concentration and reducing sugars were measured as described above. In aqueous samples, cell growth was estimated by measuring optical density at 600 nm in a Shimadzu UV-1800 spectrophotometer. A calibration curve relating OD_{600} and bacterial cell concentration (g L^{-1}) was generated by placing five 4 mL samples from the control fermentation, whose OD at 600 nm was between 0.1 and 1.5, in previously dried and weighed crucibles. The crucibles with the sample were dried to a constant weight at 37 °C and finally weighed. Then, the biomass concentration was calculated and correlated with the OD of the samples. The relationship between both parameters is shown in Eq. 7.1. The cell concentration was used to calculate the yield of cell biomass on substrate ($Y_{X/S}$) as well as to determine the maximum growth rates. X (g L^{-1}) = OD_{600} * 1.405 (Eq. 7.1).

$$X\ (g\ L^{-1}) = OD_{600} * 1.405 \quad (7.1)$$

The Folin-Ciocalteu's method (Singleton and Rossi, 1965) was used for the quantification of the total phenolic concentration. Results were expressed as mM of gallic acid equivalents (GAE).

7.2.8 Statistical analysis

The statistical analysis was conducted with the software R (version 3.4.3). A one-way analysis of variance (ANOVA) was carried out to determine whether there are statistically significant differences among the most relevant results considering a 0.05 level of significance.

7.3 Results and discussion

7.3.1 ABE fermentation with P2 medium and cellulose hydrolysate

The utilization of the cellulose hydrolysate as a substrate for ABE production involves the possibility of limited growth or restricted yields due to the presence of inhibitors. A control fermentation in synthetic P2 medium was performed for comparative purposes.

The measured pH and optical density profiles of the control fermentation (Figure 7.2A) indicated an acidogenic phase of ca. 20 h leading to acids and biomass formation. Prior to the exponential growth, which lasted up to 40 h, a short growth lag time of ca. 0.16 h allows microbial adaptation to the culture medium. The increase in pH at the end of the acidogenic phase corresponds to the solventogenic phase of ABE fermentation: organic acids together with the additional carbon source are consumed to produce final metabolites (butanol, acetone and ethanol, B, A, E, respectively). The final B concentration in the fermentation medium was about 7.4 g L^{-1} (near the inhibition threshold) while no E was detected. The ratio between ABE products at the end of control fermentation shows an increase in B production at the metabolic level. Thus, the butanol yield on glucose ($Y_{B/S}$ = 0.292 ± 0.054 g g^{-1}), was higher than the low yields for acetone and ethanol ($Y_{A/S}$ = 0.081 ± 0.013 g g^{-1}, $Y_{E/S}$ = 0 g g^{-1}). Kudahettige-Nilsson et al. (2015) reported yields of 0.24 g g^{-1}, 0.16 g g^{-1} and 0.02 g g^{-1} for B, A, and E, respectively in P2 medium with *C. acetobutylicum* ATCC 824. The biomass yield ($Y_{X/S}$ = 0.116 ± 0.019 g g^{-1}) was similar to the values reported in the literature ($Y_{X/S}$ = 0.1 g g^{-1}, Outram et al. 2016).

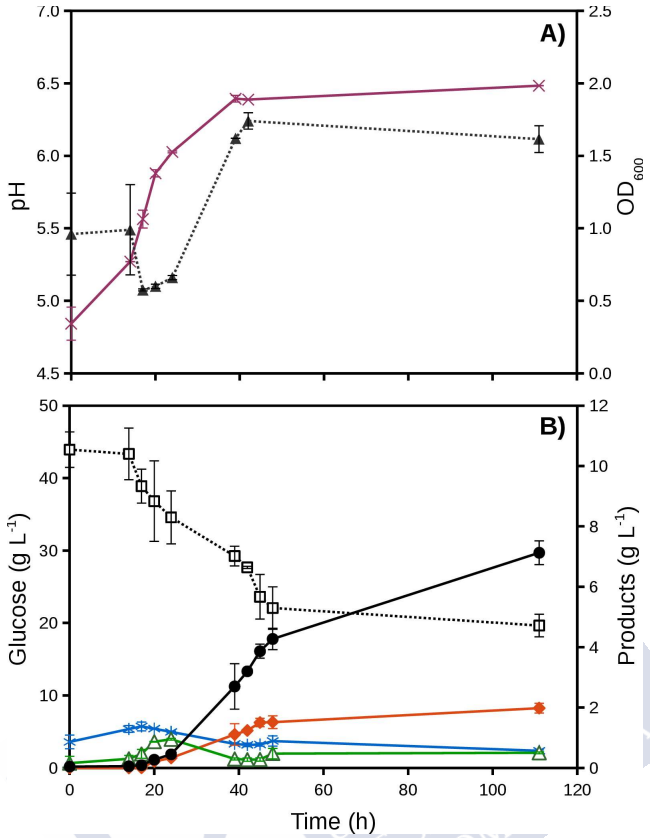

Figure 7.2. Profiles of A) pH (cross) and OD600 (full triangle), and B) glucose (void square) and products concentration (butanol (circle), butyric acid (void triangle), acetone (diamond) and acetic acid (asterisk)) during the culture of C. beijerinckii in P2 medium

The ability of *C. beijerinckii* CECT 508 to ferment the hydrolysate of the organosolv cellulose fraction was studied and the results are presented in Figure 7.3A. The maximum growth rates and growth lag times are shown in supplementary material of Gonzalez-Peñas et al. (2018). The lag time for biomass growth lasted about 29.5 h, which is significantly longer than for control fermentation in synthetic P2 medium. The hydrolysate medium potentially contains a variety of inhibitors and residual lignin, because its complete separation by centrifugation after enzymatic hydrolysis is difficult to achieve. This might hamper the process start-up and explain the increased lag time (Roth and Tipkötter 2016). The overall growth rate (0.0271 h^{-1}) was significantly lower than that of the control fermentation, with a maximum optical density attained only after 70 h of fermentation. However, similar values for the maximum concentration of bacterial cells were achieved in both fermentations

(2.79 and 2.68 g L^{-1}, for the P2 and cellulose hydrolysate media, respectively). This indicates that, after an initial adaptation of the culture, the presence of inhibitors in the hydrolysate medium did not have a negative impact on the performance of the ABE fermentation. This was also reported by Zhang and Ezeji (2012) and Ezeji et al. (2007), who reported that the addition of furfural and HMF below the threshold of 2-3 g L^{-1} had a positive impact on the overall growth of *C. beijerinckii* BA101, because of the conversion of the inhibitors into less toxic compounds.

Figure 7.3B shows the time profile of reducing sugars concentration in the fermentation broth, together with the main metabolites. Interestingly, the rate of sugar consumption was slightly higher (7.5%) in the hydrolysate medium compared to the synthetic P2 medium. Qureshi et al. (2007) have previously reported a significant increase of r_{sugars} (82.5%) in ABE fermentations with *C. beijerinckii*, when changing from a synthetic medium to a wheat straw hydrolysate based medium.

The highest rate of ABE production was observed after 40 h of fermentation, although solvent formation started earlier. The presence of considerable amounts of acetic acid in the cellulose hydrolysate medium (more than 6 g L^{-1}) may help to enhance final product yield, as it can be partially converted into solvents, hence triggering the solventogenic phase (Tipkötter et al. 2014; Chen and Blaschek 1999). Moreover, according to the data depicted in Figure 7.2B, both metabolic pathways (acidogenic and solventogenic) seemed to occur simultaneously, as the highest production rate of B and butyric acid occurred between 60 and 80 h of fermentation. The existence of two parallel butanol formation pathways (direct route and classical *Clostridial* acid assimilation route) was proposed by Jang et al. (2012) based on metabolic engineering studies. The final butanol concentration reached in this study (7.65 g L^{-1}) was close to the inhibitory threshold. It is known that B concentrations higher than 8 g L^{-1} causes cell membrane fluidity and progressively stops metabolic activity (Jones and Woods 1986). The final concentrations of A and E were 4.30 and 1.38 g L^{-1}, respectively, i.e. nearly the expected product ratio was found. The total ABE concentration (13.33 g L^{-1}) was 40% higher than the total titer measured in the P2 control fermentation. This is also in accordance with the previously published literature (Roth and Tipkötter 2016).

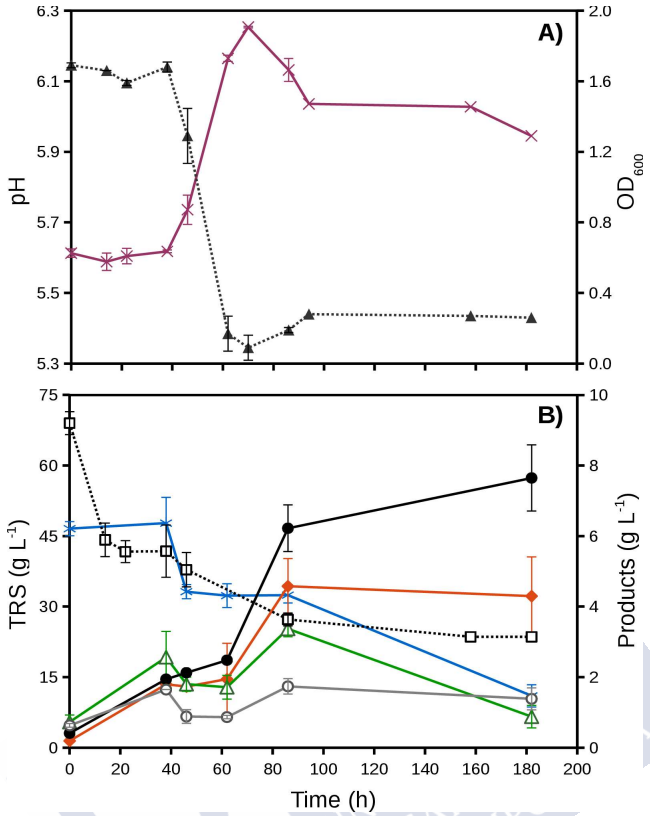

Figure 7.3. Profiles of A) pH (cross) and OD600 (full triangle), and B) TRS (void square) and products concentration (butanol (circle), butyric acid (void triangle), acetone (diamond) and acetic acid (asterisk)) during the culture of C. beijerinckii in hydrolysate medium

The results indicated that the carbon source in synthetic P2 medium can be replaced by a fraction of hydrolyzed organosolv cellulose without reducing the final solvent titer. In both cases, glucose consumption and metabolic activity was maintained until the inhibitor B concentration threshold was approached. Nevertheless, the hydrolysate medium increased the growth lag time of the culture, and solvent formation seemed to be delayed over the control fermentation. This is in line with Roth and Tipkötter (2016), who compared the performances of batch fermentations using synthetic media and hydrolyzed cellulose fibers from pre-treated beech wood, reaching a maximum B concentration and solvent titer of 7.2 g L^{-1} and 0.2 g g^{-1} sugar, respectively. The quoted authors observed that a detoxification process resulted in a reduced ABE production. Organosolv pretreated beech wood hydrolysate was also used as a substrate for B fermentation in Tippkötter et al. (2014). A total solvent (ABE) concentration of 15.1 g L^{-1} was

achieved, and improved growth parameters and higher B yield were observed, when pure sugars were substituted by the hydrolysate. Amiri and Karimi (2015) studied the suitability of an organosolv pretreatment for ABE production from pine and elm and observed an increased ABE titer from 5.5 (untreated wood) to 11.6 g L^{-1}. Zhu et al. (2015) proposed an integrated fermentation process based on organosolv pretreated reed for biobutanol production and obtained final titers of 9.1 g L^{-1} butanol and 14.2 g L^{-1} ABE.

7.3.2 Extractive fermentation with P2 medium and cellulose hydrolysate

Extractive fermentations were carried out in duplicate batches with P2 and a medium based on cellulose hydrolysate for comparative purposes. Figure 7.4 displays the time profile of pH, optical density (a), and the concentration of glucose and main metabolites (b) in the extractive P2 fermentation medium. The change from conventional to extractive fermentation increased growth lag time from 0.16 to 33.83 h, while the maximum growth rate decreased from 0.0651 to 0.0145 h^{-1}, as shown in supplementary material of Gonzalez-Peñas et al. (2018). In solventogenic phase, pH increased to a less extent than non-extractive fermentation, probably because of butanol extraction. The maximum biomass concentration in extractive fermentation was 36% lower than in control fermentation with the same medium, which can be attributed to mid-term toxicity of 2B1O. Kollerup and Daugulis (1985) classified the modes of cell inhibition in extractive fermentations into different mechanisms depending on the initial effect of the solvent on the metabolic and enzymatic activity during the fermentation. Mid-term toxicity against *C. beijerinckii* has already been observed by Perez-Bibbins et al. (2017). The total concentration of each solvent (the sum of solvents from the aqueous and organic phases) increased over conventional fermentation control, adjusting the total final B concentration to 11.24 g L^{-1}, which is 35% higher than that of the conventional fermentation, and the total ABE titer increased by 95%. However, the solvent production rate was significantly reduced after 160 h of fermentation, despite the low concentration of aqueous B (< 4 g L^{-1}), which is far from the inhibition threshold, and the high glucose concentration was maintained in the fermentation broth.

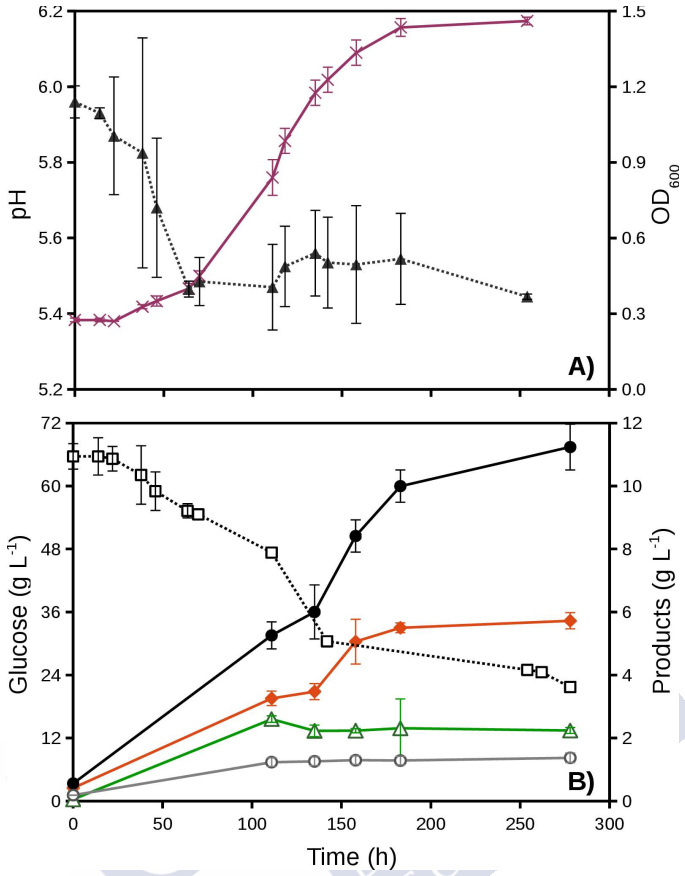

Figure 7.4. Profiles of A) pH (cross) and OD600 (full triangle), and B) glucose (void square) and products concentration (butanol (circle), butyric acid (void triangle), acetone (diamond) and ethanol (void circle)) during the culture of C. beijerinckii in extractive fermentation with P2 medium

Figure 7.5 shows the variation of the different cell forms during conventional and extractive fermentation, together with the concentration of aqueous B in the broth. The evolution of the percentage of sporulating forms is parallel to that of aqueous B in conventional fermentation, so its presence increases from the onset of the solventogenesis. However, in extractive fermentation, sporulation started to increase after 150 h of fermentation. At this point, aqueous butanol concentration was well below the inhibitory threshold (~4 g L^{-1}) and remained unchanged. The increase in sporulating forms coincided with the decrease in metabolic activity and is likely to be attributed to mid-term toxicity effect of 2B1O present in biphasic reactors. González-Peñas et al. (2015) have observed an increase in the number of spore cells with extractive fermentation 2B1O in a laboratory scale bioreactor.

However, they observed a significantly higher percentage of sporulating cells, which could be due to an enhancement in the mass transfer between the liquid phases (higher 2B1O concentration in the aqueous phase) due to the bioreactor configuration (high L/D, mechanical agitation) compared to the flask fermentations carried out in the present study.

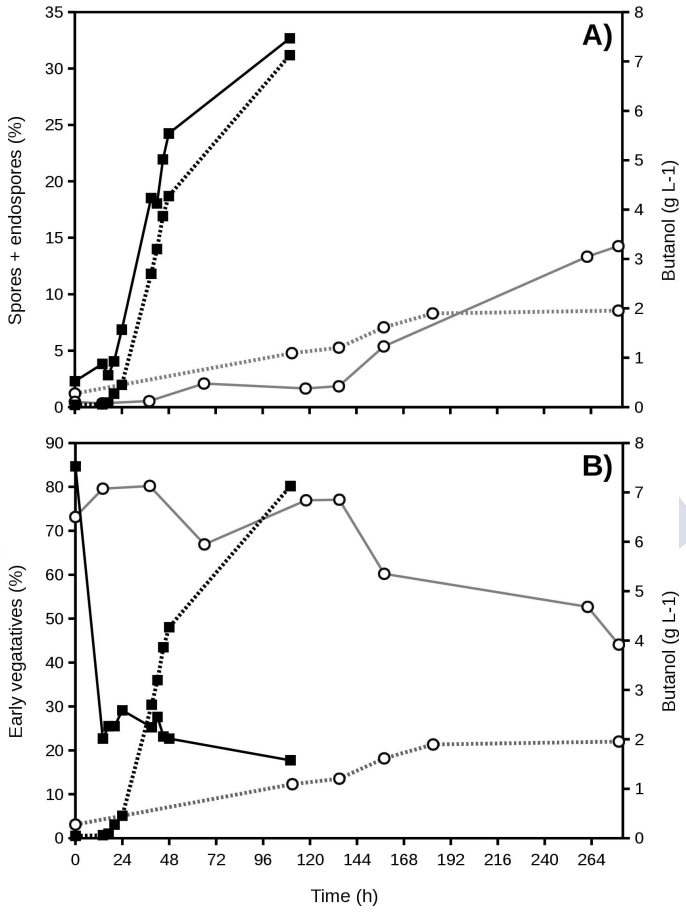

Figure 7.5. Population Dynamic of different types of cells of C. beijerinckii. Percentage of sporulating (A) and vegetative (B) cells during batch conventional (full square) and extractive (open circle) ABE fermentations. Dotted line shows the concentration of butanol in aqueous phase

Total glucose consumption in extractive fermentation increased compared to conventional fermentation In the case of extractive fermentation with cellulose hydrolysate based medium (Figure 7.4 and supplementary material of Gonzalez-Peñas et al. (2018)), the lag time in biomass growth was greater than that observed in the conventional fermentation with the same medium and without ISPR (62.50

and 29.53 h, respectively), i.e. not only the presence of inhibitors or residual lignin may have a negative effect, but also the extracting solvent may inhibit the initial bacterial growth. The rate of sugar consumption (r_{sugars}) was also affected by the presence of 2B1O, which was reduced from 0.485 to 0.251 g L^{-1} h^{-1}, when changing from the conventional fermentation to extractive fermentation in the same medium. Similarly, when 2B1O was used with P2 medium, r_{sugars} was reduced from 0.451 to 0.248 g L^{-1} h^{-1}. Thus, the reduction of r_{sugars} was similar in both media (51.8 and 54.5%, respectively).

As in the case of extractive P2 medium fermentation, the acidogenic phase lasted longer, and pH continued to decrease until the end of the fermentation, probably due to a higher accumulation of acids in the aqueous phase and the concomitant extraction of B into the organic phase. The exponential growth rate was lower than that of conventional fermentation without ISPR (0.0108 and 0.0271 h^{-1}, respectively), and so the maximum optical density. Extractive fermentation with hydrolysate-based medium reached lower bacterial growth than with P2 based medium (1.52 and 2.05 g L^{-1}, respectively). The time profile of the products for cellulose hydrolysate extractive fermentation is shown in Figure 7.6B. Again, the total solvent concentration increased over the conventional culture, indicating that inhibition was, at least, partially alleviated. But in this case, only B concentration was increased (from 7.65 to 9.22 g L^{-1}), A and E concentrations remained unchanged (with 4.26 and 1.27 g L^{-1}, respectively). Again, B production seemed to stop after 160 h of fermentation, despite the low aqueous B concentration (<4 g L^{-1}) and this could be attributed to the mid-term toxicity of the solvent.

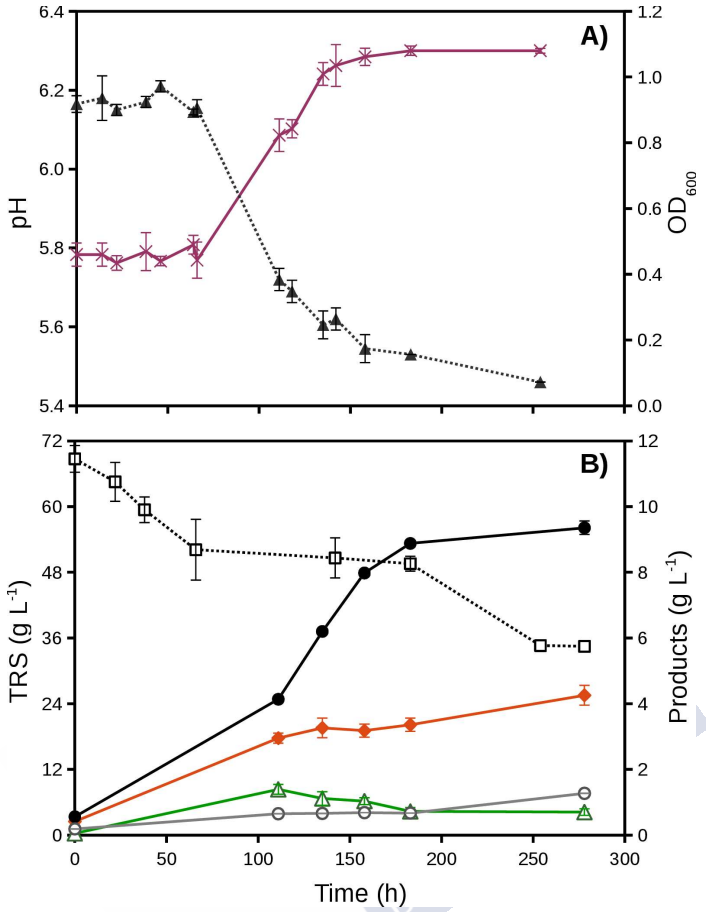

Figure 7.6. Profiles of A) pH (cross) and OD600 (full triangle), and B) glucose (void square) and products concentration (butanol (circle), butyric acid (void triangle), acetone (diamond) and ethanol (void circle)) during the culture of C. beijerinckii in extractive fermentation with cellulose hydrolysate medium

There is scarce literature concerning *in situ* extractive ABE fermentations in complex media. This technology has been applied to organosolv hydrolysate of spent mushroom substrate with biodiesel as an extractant (Zhu et al. 2016). Butanol production increased to 14.65 g L^{-1}, i.e. almost by 22% compared to the conventional batch. Hence, the improvement achieved with the extractive fermentation was like the one attained in this work. To explore the capability of semi-continuous B fermentation with *in situ* removal, the fed-batch fermentation with sequential feeding of the hydrolysate was carried out leading to a maximum B concentration of 30.21 g L^{-1}. In our case, the use of 2-butyl-1-octanol in fed-batch

fermentations with *Clostridium beijerinckii* is discouraged due to its probable mid-term toxicity.

Based on the statistical analysis, it can be concluded that B yield in extractive fermentation on cellulose hydrolysate medium (0.258 ± 0.016 g g^{-1}) was significantly higher (p = 0.006) than the one obtained in conventional fermentation in the same medium (0.159 ± 0.028 g g^{-1}). However, the final B titers of these fermentations were not significantly different (p = 0.091). The highest B concentration was obtained in the extractive fermentation in P2 medium (11.24 g L^{-1}), however, its B yield was not considerably different than those reached in the conventional fermentation in P2 medium and in the extractive fermentation with cellulose hydrolysate (p > 0.24). On the other hand, no statistical differences were found in the B concentrations reached in the conventional fermentations with both media (p = 0.5485). The lowest B yield was obtained in the conventional fermentation with cellulose hydrolysate, while the B yields of all other fermentations were similar.

7.3.3 Optimization of the operating conditions

Comparative fermentations carried out in the first part of this work validated the use of organosolv hydrolysate as a potential 2G substrate in ABE fermentation. However, even when fermentation profiles of the base case presented typical biphasic and end-product inhibition trend, the maximum solventogenic capability of the strain was not reached, since solvent production ceased before "classical" inhibition threshold of butanol (around 10 g/L (Jones and Woods, 1986)). To boost the performance of the ABE fermentation up to the expected final butanol concentration two operational approaches were investigated: a) the addition of Calcium to the medium and b) the modification of the culture temperature.

a) Calcium addition into the medium

Recent studies revealed that the presence of Calcium in the fermentation broth preserves the solventogenesis and alleviates product inhibition. This is not only due to the buffering capacity of $CaCO_3$, in agreement with Qi et al. (2017), but also to its beneficial effect to influence key cellular processes (Han et al. 2013). These authors observed the increase of key enzymes responsible in the clostridial solventogenic pathway, sugar transport or butanol tolerance. In that work, cell growth, glucose utilization, ABE titer and yield were significantly increased when at least 4 g/L of $CaCO_3$ was supplemented to the standard P2 medium with *Clostridium beijerinckii NCIMB 8052* .

According to literature, P2 standard medium was supplemented with 6 g/L of $CaCO_3$ (P2-Ca medium) and differences in terms of glucose utilization, butanol and butyric acid were quantified according to serum-bottle protocol described in M&M

section. The experiments were carried out in duplicate and the temperature was fixed at 37 °C. As depicted in Figure 7.7, glucose utilization was 2.3 times higher in P2-Ca based fermentation compared to those carried out on standard medium. Butanol and acetone production were also enhanced in the P2-Ca medium (7.6 g/L compared to 1.8 g/L), while ethanol production was negligible. Therefore, modified P2-Ca medium was chosen to be utilized for subsequent experiments.

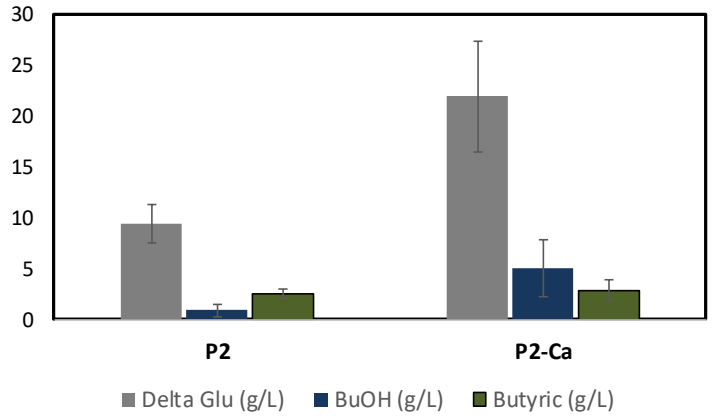

Figure 7.7. Effect of Calcium addition on ABE fermentation production with C. beijerinckii

b) Temperature

One possible explanation for low-solvent producing ABE fermentations is the "acid-crash" phenomenon. When this occurs, excess of acid production is observed, while not significantly shift to solventogenic phase is appreciated (Maddox et al. 2000). Sometimes solventogenic phase starts, but after a short time, all metabolic activity cease. Maddox et al. (2000), forced the "acid-crash" to occur in several batch ABE fermentations, to identify the responsible variables and to preconize practical operating conditions to prevent it. According to the authors, a too high acid production rate in the early stage of fermentation could be partly responsible of acid-crash. In fact, considering the solventogenic phase as a detoxification step (of toxic undissociated butyric acid mainly), a too quick accumulation of acid would induce solventogenic to start earlier but acid production would go on and finally reach too high level leading solvent product and metabolic activity to completely cease. In this sense, any variable allowing to control initial acid kinetics might help to prevent "acid crash". One of them is the temperature. In order to explore this possibility, three temperatures (30, 34 and 37 °C) were tested in triplicates serum-bottles experiments with P2-Ca modified medium.

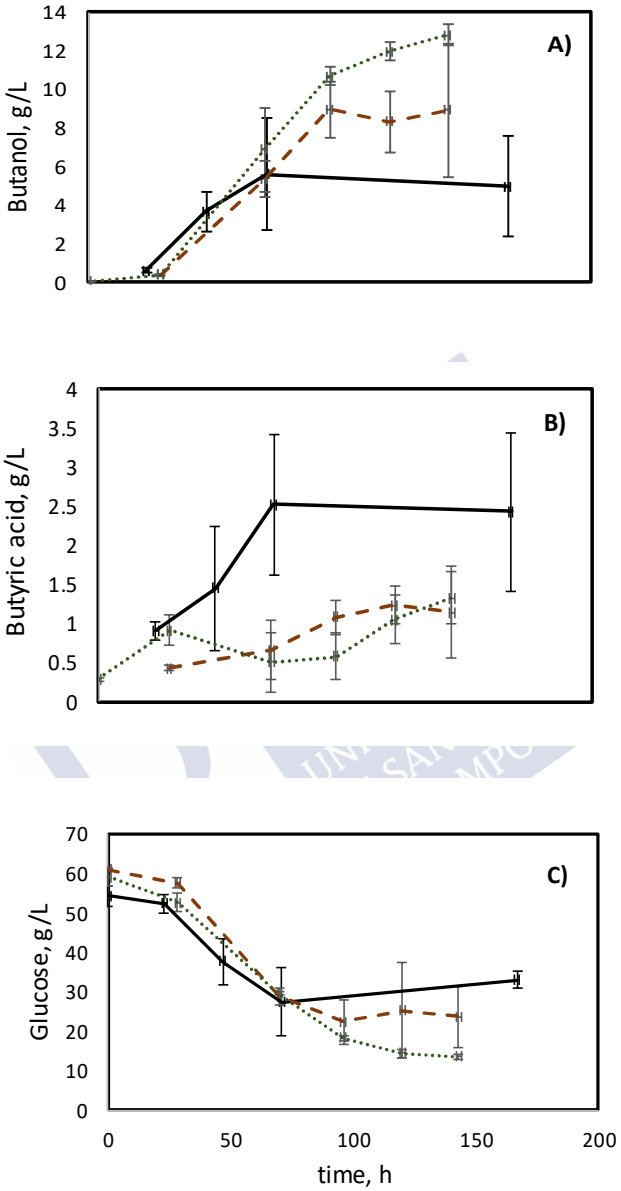

Figure 7.8 Profiles of A) butanol, B) butyric acid and C) glucose during culture of C. beijerinckii in P2 medium at 30 °C (dotted line), 34 °C (dashed line) and 37 °C (continuous line)

Figure 7.8 depicts the time evolution of glucose, butanol and butyric acid, which reveals that the "acid-crash" seems to be prevented by lowering the culture temperature. At 37 °C, butyric acid was produced until the cessation of metabolic activity at 70 h (maximum concentration of 2.4 g/L). Solventogenic step was initiated at 20 h, reaching a final butanol production of 5 g/L. At 30 °C, glucose consumption was more than 2 times higher than at 37 °C, and butanol concentration reached 12.7 g/L showing the best product-tolerance. A clear biphasic profile was appreciated, maintaining a maximal product rate between 30 and 100 h of fermentation, concomitantly with acid consumption. From 100 h, cycling production of acid and solvents occurred. An intermediary situation was observed at 34 °C. Acid and solvent production followed parallel trends until cell activity completely cessed at about 100 h. The final butanol titer at 34 °C was 8 g/L, showing a significant solventogenic activity, but still lower than the expected inhibition threshold.

Based on these results, it was decided to fix the fermentation temperature to 30 °C in further experiments. It was expected that the combination of lower temperature and the presence of Ca in the medium might help to prevent "acid crash".

7.3.4 Enzyme detoxification of cellulose hydrolysate by laccase

The detoxification of organosolv cellulose hydrolysate by laccase treatment prior to ABE fermentation was evaluated through two different scenarios:

- A solid-liquid separation step (centrifugation) is applied after the enzymatic hydrolysis to remove the remaining solids prior to laccase treatment (HC)
- The laccase step is applied directly to the hydrolysate issued from enzymatic hydrolysis, without any previous operation (HCB, black-cellulose hydrolysate). Regarding to further scale up of the process, the direct fermentation after enzymatic hydrolysis would alleviate investment cost and simplify the process scheme (as in the case of 2G-ethanol).

The conditions of the laccase treatment are presented in Table 7.1. An incubation time of 24 h was considered. Applied operation conditions reached a compromise between enzyme activity and stability. As can be seen in Table 7.2, initial total phenol content is higher when solid/liquid separation is not applied (10.4 mg GAE/g cellulose compared to 4.6 mg GAE/g cellulose), but its removal is significantly lower. Garcia-Torreiro et al. (2017) proved that phenolic compounds from the residual lignin in the pulp could be released into the medium during the 24 h enzymatic treatment. In this case, real effective percentage removal of total phenols from HCB might be higher, since its estimation considered only the initial measured concentration of total phenols.

Table 7.1. Laccase treatment of cellulose hydrolysate with and without S/L separation (HC and HCB respectively)

Substrate	*Solids (%)*	*Temperature (°C)*	*Enzyme dose (U/L)*	*Total initial phenols (mg GAE/g cellulose)*	*Removal of total phenols (%)*
HC	10 (w:w)	30	4000	4.6	35
HC-Black	10 (w:w)	30	4000	10.4	7.8

a) Solid-liquid separation after enzymatic hydrolysis

Figure 7.9 presents the profiles of the fermentations with the hydrolysate-based media with and without laccase detoxification (HC-Lac and HC, respectively), together with the P2-Ca base medium as control. Synthetic P2-Ca medium contains the same sugar concentration as the hydrolysate, and 85% of this initial glucose was consumed within 140 h (48.2 g/L of glucose consumed). In the hydrolysate medium, glucose was depleted (39 g/L) and the other reducing sugars were also consumed (84 and 72% of consumption for HC and HC-Lac respectively), leading to equivalent total sugar consumptions in both media (P2-Ca and hydrolysate). In conformity with the results obtained in the first part of this work, an increased lag time was observed during sugar consumption when hydrolysate-based fermentation is compared to P2-Ca based medium. This delay was also appreciated in solvent production course. In synthetic medium experiment, maximal butanol and total ABE production of 12 g/L and 16.2 g/L respectively were achieved, but volumetric productivity decreased after 100 h of fermentation. In the hydrolysate-based fermentation, solventogenesis started at 60 h and it carried on until sugar depletion. Maximum butanol and ABE concentration attained 11.8 g/L and 16.87 g/L in the HC fermentation, while 9.97 and 14.14 g/L (18 and 19 % lower) were reached with the HC-Lac medium. Since 35% of total phenols were removed during the laccase treatment, it can be concluded that they were not responsible of the increased lag-time observed between synthetic and centrifuged HC-based medium fermentation.

Butyric acid production level was significantly lower in hydrolysate-based medium compared to P2-Ca based medium. In fact, the initial concentration of acetic acid in the hydrolysate medium is at least two-fold that of the P2-Ca medium. Besides, acetic acid has been stated to be one factor in triggering solventogenesis.

Bellido et al. (2018) studied the synergistic effect of the different inhibitory compounds present in hydrolysate-based substrate to ABE fermentation and concluded that the inhibition by phenolic compound can be overcome by addition of organic acids.

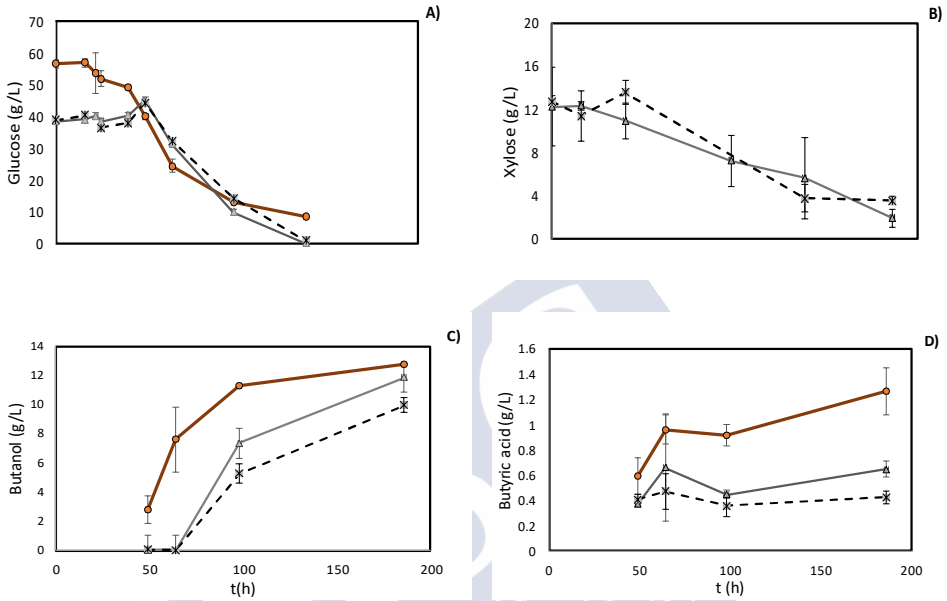

Figure 7.9. Profiles of A) glucose, B) other reducing sugars, C) butanol and D) butyric acid during culture of C. beijerinckii in P2-Ca medium (orange circle), HC (grey triangles), and HC with Laccase treatment (dashed line)

b) No solid-liquid separation after enzymatic hydrolysis

Additional comparative experiments were carried out to assess the effect of a laccase detoxification step prior to fermentation of black cellulose hydrolysates that contain solids (HCB). The profiles of laccase treated, and non-treated black-hydrolysate based fermentations (HCB-Lac and HCB, respectively), as well as that of the P2-Ca based control fermentation, are depicted in Figure 7.10. After a lag-time of about 50 h, both glucose and other reducing sugar were consumed up to 85 and 95% in the case of HCB and HCB-Lac, respectively.

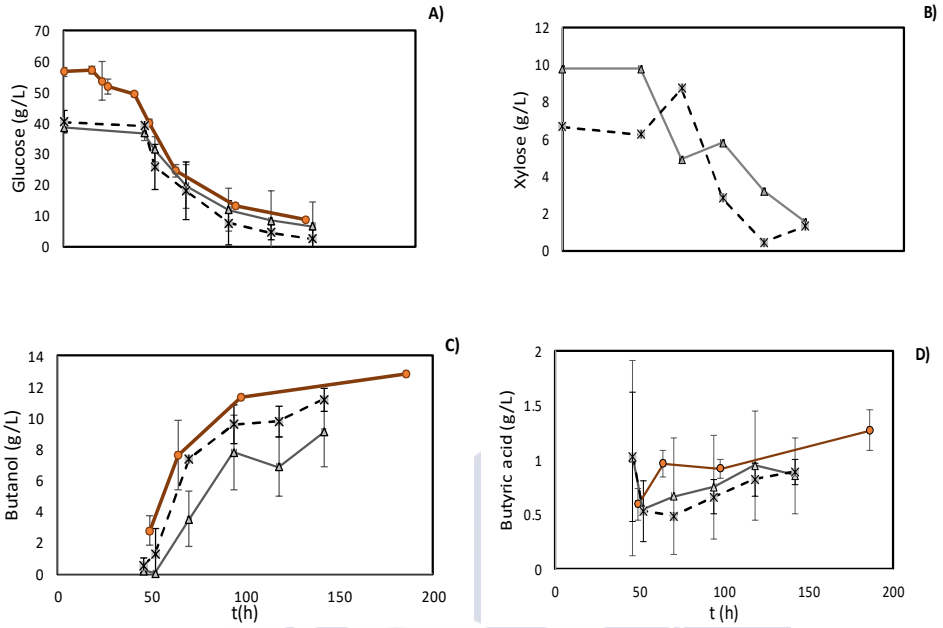

Figure 7.10. Profiles of A) glucose, B) other reducing sugars, C) butanol and D) butyric acid during culture of C. beijerinckii in P2-Ca medium (orange circle), HC-Black (grey triangles), and HC-Black with laccase treatment (dashed line).

Solventogenesis was fully achieved in all configurations and total butanol production reached 11.2 (0.7) and 9 (2.1) g/L in HCB fermentations with and without laccase detoxification, respectively. Total ABE production with HCB-Lac was 15.9 g/L (0.99) which is 24% higher than that reached with the HCB without laccase treatment. From these data, laccase treatment seems beneficial when no solid-liquid separation was achieved after enzymatic hydrolysis, even if not more that 7% of total phenols are apparently removed prior to ABE fermentation. As discussed below, lignin is disposed in droplets that are strongly attached to organosolv cellulose fibers (Garcia-Torreiro et al. 2017), and some lignin might be released into the medium during laccase treatment, increasing the fraction of dissolved phenolic compounds in the broth. In that case, the effective total phenolics removal might be higher than 7%. Moreover, initial total phenolics concentration measured in black hydrolysate was 26% higher than the critical toxic concentration for ABE fermentation reported in Bellido et al. (2018).

In a previous work dealing with ABE fermentation of hydrolysate of cellulose fraction from organosolv pretreatment, the remaining solids were completely separated from the medium by filtering through 0.2 µm membranes (Roth and

Tippkötter, 2016). The present study demonstrates that solid-liquid separation prior to ABE fermentation is not necessary. No differences in the maximal butanol concentration were observed after the laccase treatment of the hydrolysate with solids ($p = 0.252$). On the contrary, butanol titer was significantly reduced after applying laccases to the hydolysate without solids ($p = 0.003$). Specific disposition of lignin droplets into the cellulose fibers (García-Torreiro et al. 2018) might favor cells adhesion to the surface, creating a microenvironment for *Clostridium* biofilm development that is propitious to ABE fermentation. This aspect deserves to be further investigated, since the elimination of the solid-liquid separation after enzymatic hydrolysis step would alleviate investment cost and simplify the process scale-up.

7.4Conclusions

Cellulose hydrolysate from organosolv pretreated beech wood proved to be a suitable, abundant, and economical available substrate to produce acetone (A), butanol (B) and ethanol (E) by *C. beijerinckii* CECT 508 fermentation. Comparative fermentations showed that the total solvent production increased by 40% in the hydrolysate medium compared to the control, and this is enhanced by 10% with extractive fermentation with 2-butyl-1-octanol. However, the presence of remaining inhibitors from the organosolv pretreatment probably increased the lag time in fermentations with hydrolysate.

To solve this drawback, a laccase detoxification treatment prior to ABE fermentation was evaluated on cellulose hydrolysates with and without remaining solids from the enzymatic hydrolysis. It was concluded that solvent production was fully achieved in all configurations, and surprisingly, final ABE titer was enhanced by 25% in the medium with remaining solids. In this case, the application of the laccase treatment increased the total solvent production by 24%. Regarding to further scale up of the process, the direct fermentation after enzymatic hydrolysis would alleviate investment cost and simplify the process scheme. These results open a potential improvement vector for 2G based ABE biorefinery, which deserves to be further investigated.

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8. General discussion and conclusions

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OUTLINE

8.1 Main outcome and positioning of this work

Today, the production model is being redefined in favor to a bioeconomic based system. To progressively replace the fossil-based fuels and products (materials, bulk chemicals), this transition will certainly come across with the development of new bio-based processes through the application of modern white biotechnology. But here is also the opportunity for the "revival" of old industrial bioprocess that were abandoned in the past, when they could no longer compete with the emerging petrochemistry.

The Acetone-Butanol-Ethanol (ABE) fermentation was the 2nd industrial fermentation until the mid of the 20th century. After decades of complete cessation, this process is today subject to a renewal interest, as it is reflected in the increasing number of R&D projects, publications and patents. However, to make this process competitive, it is necessary to face several issues that strongly affect the production costs. In other words, the old version of the ABE process might be "intensified" prior to entering again the market.

First, ABE fermentation is fully governed by end-product (mainly butanol) inhibition. In conventional process, final aqueous broth is extremely diluted, which implies too much expensive downstream and a huge amount of water turnaround. Therefore, the application of an In-Situ Recovery Technique to alleviate product toxicity has led the research around ABE intensification for the last decades. Among the investigated ISPR techniques, liquid extraction offers some important advantages, like its well-known basic principles and extensive applications.

Gaining a better understanding of the ABE extractive fermentation and the development of strategies to fully exploit the features of the extraction solvents have been the main objectives of this research. To accelerate the development of the integrated process, the comprehension of the key associated phenomena, with special focus on ABE-solvent interactions, is necessary. In this sense, this thesis offers a complementary view framed within the methodological scheme depicted in Figure 8.1.

A global screening methodology (Chapter 2) allowed to classify the potential solvents according to physical and biological characteristics. The main outcome of Chapters 3 and 4 is the enhancement of the understanding of the ABE extractive fermentation process. To accomplish it, product kinetics and microbial population dynamics in extractive configurations, with solvents of different nature were investigated. Chapter 5 deals with ABE model development, with the aim of translating previously acquired knowledge into mathematical expressions.

Secondly, in a more process-oriented view, a comparative techno-economical assessment (Chapter 6) was developed based on experimental performance parameters. The aim is to prospectively analyze the main cost distribution, and to identify bottlenecks. As a result, the integrated techno-economic assessment of the process comprised fermentation, regeneration and separation sections, and was based on solvent-specific performances. It serves as a useful guideline to select process conditions and to orientate further research. Finally, the use of an organosolv cellulose hydrolysate as a substrate is evaluated in Chapter 7. This last part is oriented to develop alternatives to the sustainable ABE production within a 2G biorefinery.

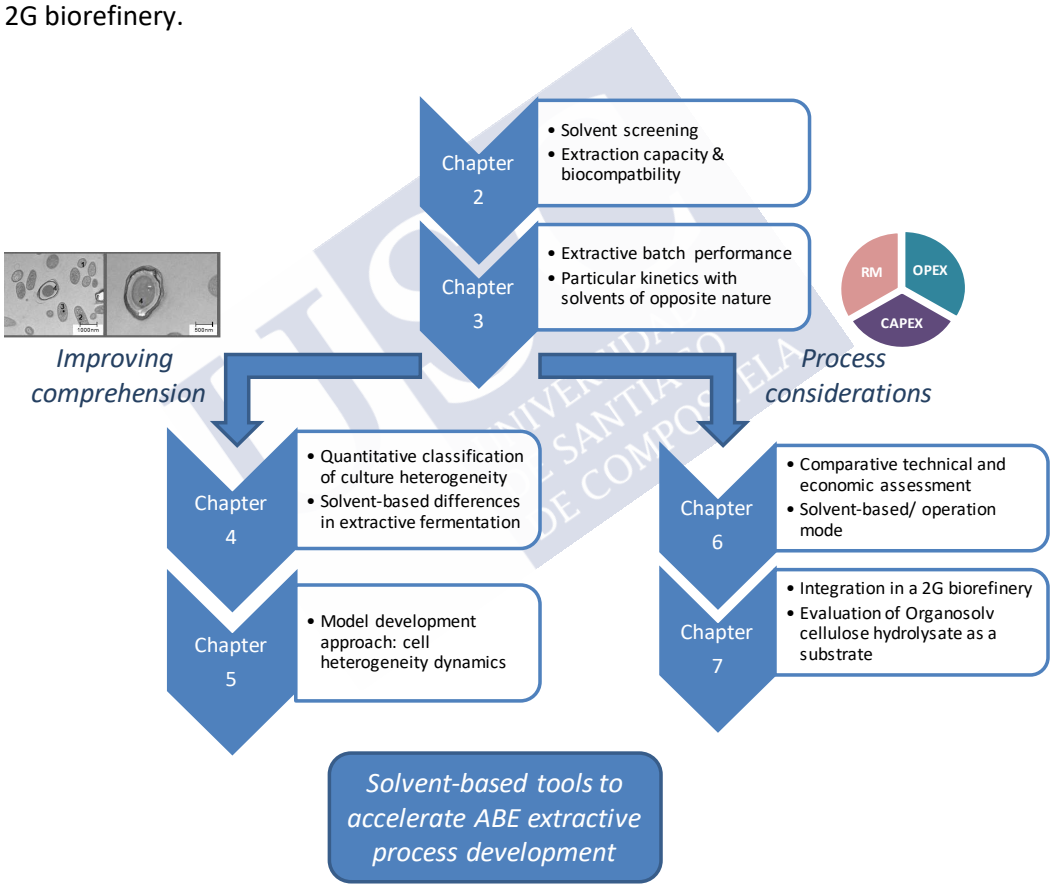

Figure 8.1. General scheme and summary of the main content of the Thesis.

8.2 Solvent selection for ABE extractive fermentation

Solvent selection for ABE extractive fermentation was extensively studied during the early 90s (Malinowski and Daugulis, 1994, Groot et al. 1991). Most of these works focused on finding an extracting agent fulfilling both criteria: optimal butanol partition coefficient and biocompatibility towards Clostridia. Oleyl alcohol (OA) has been the reference solvent since it offers a relatively high butanol partition coefficient (about 3 g/g), and it resulted harmless in ABE fermentation (Roffler et al. 1986).

A global screening methodology was applied (Chapter 2) to classify the different chemical families according to their ABE products extraction capacity and to the biological response provoked in the microorganism (biocompatibility and bioavailability evaluation). The optimal solvent resulted from a compromise between different criteria. For instance, in most cases, butanol partition coefficient (which determines the quantity of solvent required for the extraction) varies inversely to selectivity (which expresses the quantity of water that would be removed concomitantly, affecting subsequent regeneration step). Even more critically, when analyzing the whole group of solvents tested, the extraction capacity is opposite to biocompatibility. In agreement with Daugulis et al. (1991), asymptotic behavior is obtained when representing the logP value of the different solvents (as an indicator of toxicity) against metabolic activity in an extractive fermentation. The asymptote region (logP values between 4 and 6) englobe those solvents presenting the best extraction features, but that might present some degree of toxicity or inconsistent behavior in repeated experiments (Offeman et al. 2008)

Two solvents with opposite characteristics were selected to be further investigated: a) a vegetable oil (pomace oil) that present low partition solvent (< 1 g/g) but assured biocompatibility, and b) a C12-based Guerbet alcohol (2B1O), that has never been previously tested in ABE fermentation. 2B1O offers the highest partition coefficient for butanol (> 6.5) but falls in the “critical” region of toxicity. The question was whether it could be interesting to work with a solvent close to the toxicity threshold, to benefice from best extraction capacity, or if, on the contrary, it would be advantageous to guarantee full long-term biocompatibility, in detriment of the partition coefficient. But, prior to perform a prospective global assessment (Chapter 6) some unexpected experimental results deserve to be further analyzed.

Butanol yield was systematically higher in extractive fermentations with 2B1O. In Chapter 3, quantitative distribution of the two possible metabolic pathways for butanol production was estimated from molar acetone equivalents (Jang et al.

2012). Only 35% of butanol was obtained concomitantly to acids uptake and acetone production (while 65% is obtained by the "direct way"), instead of 75-80% in the case of control or vegetable oil based extractive fermentation. The reason behind this metabolic modification must be further investigated, and some hypothesis were discussed in Chapter 2 and Chapter 3. Butanol yield in extractive fermentation was correlated with butanol and butyric acid partition coefficients. Moreover, our data (Chapter 3) showed that the organic phase acted as a source of butyric acid during the solventogenesis (previously extracted during acidogenesis). It has been reported that butyric acid addition during solventogenic phase enhances butanol to acetone ratio (Tashiro et al. 2004, Li et al. 2014). In addition, acidic conditions favor solventogenic behavior and butanol metabolic pathway is stimulated at low pH (Li et al. 2011). Butyric acid from the organic phase would have created NADH pressure by lowering the pH after the metabolic switch. This redox imbalance would enhance product yield, since oxidation of NADH is coupled to butanol and ethanol production, but not to acetone's. This complementary hypothesis is not excluded from our data, particularly from pH profiles in 2B1O based extractive fermentation that did not present the expected minimum observed in uncontrolled batch ABE fermentations. It can be concluded that the presence of certain solvents (close to the toxicity threshold) might alter Clostridia response in batch process, beyond what would be expected from separation/extraction issues.

While butanol yield was improved, volumetric productivity was degraded in some of the performed extractive fermentations with 2B1O. On the other hand, volumetric productivity was maintained in VO-based extractive fermentation, despite butanol inhibition relief.

Two types of toxicity can be distinguished (Salter et al. 1995): "dissolved" (when the microorganism is exposed to subsaturation concentrations of the solvent) or "biphasic" (created by the presence of an interface between aqueous and organic phase). The first contribution has been related with a critical solvent-independent concentration at the cell membrane, which may affect the modification of the membrane composition, modulation of the enzymatic activity or the permeabilization of the membrane (Salter et al. 1995, Daugulis,1997). Among the mechanism argued to explain the effects of "biphasic toxicity" are the possibility of cell coating and attraction to the interface (thus limiting nutrient diffusion from the medium), or disruption of the cell wall, causing leakiness and extraction of some essential cellular components (Kollerup and Daugulis, 1984; Salter et al. 1995).

If all the experimental results of the extractive fermentations at different lab-scales are analyzed together (Table 8.1), some trends are revealed. Mass transfer is strongly improved when passing from small scale test tubes used in

biocompatibility assays (Chapter 2) to stirred tank biphasic reactor (Chapter 4). In fact, not only L/D but also agitation is enhanced when sizing up. However, despite more advantageous conditions for mass transfer between phases, equilibrium approach of butanol was already high in screening test (65%), and it attains 100% in both sealed flask and stirred tank reactor. Thus, it can be deduced that 2B1O fermentations productivity degradation observed in scale up is caused mainly by biphasic toxicity (since dissolved 2B1O contribution might not differ in both configurations).

On the other side, VO-based extractive fermentations did not present any volumetric productivity variation over control at different scales (Table 8.1). This solvent has negligible solubility in the water phase, however, when put in close contact with the microorganism at the interphase it cannot be excluded that some degree of partitioning into the cell's membrane (lipidic structure) occurs. This effect might counterbalance the benefit of removing toxic butanol by liquid extraction, thus explaining why volumetric productivity did not improve despite solvent biocompatibility.

Table 8.1. Butanol results in extractive fermentation at different lab-scales.

		Solvent Screening (CHAPTER 2)	**Batch dynamics (CHAPTER 3)**	**Culture heterogeneity (CHAPTER 4)**
				
% ΔYield (over control)	2B1O	+30%	+46%	+35%
	VO	+0	+0	-12%
%Δ Titer (over control)	2B1O	+62%	+100%	+118%
	VO	+30%	+60%	92%
%ΔProductivity (over control)	2BO	-	0 %	-22%
	VO	-	0%	0%
Mass transfer conditions		High L/D. No agitation	Sealed lab flask. "Soft" agitation (shaker)	Low L/D. Mechanical stirring (both phases)
Equilibrium approach (%)		65%	100%	100%

Biphasic toxicity is expected to increase during the bioprocess scale up, since a higher L/D in industrial reactors and the strongest agitation rate to keep cells in suspension and achieve proper mixing times, would lead to enhance surface interphase. However, when phase toxicity is predominant, the bioreactor technology may be adapted to control or minimize this effect. The results obtained in this work (Table 8.1) seem favorable to the development of biofilm-based bioreactor for in situ extractive fermentations. It has been already reported that

immobilization protects cells against solvent in extractive ethanol fermentation (Airas-Barros et al. 1986). In this paper, the diffusional and steric limitations are proposed as the main protection factors.

8.3 Culture heterogeneity and modeling issues

In this work, special attention was put on the dynamics of the Clostridia cell cycle and the physiological response to the environmental factors during a batch or fed-batch process. Flow cytometry resulted a useful technique to monitor the morphological changes of the culture occurring during ABE control and liquid-liquid extractive fermentation with solvents presenting different polarity. Besides, it was found that in fed-batch ABE extractive fermentation with pomace olive oil, a continuous butanol production can be achieved by predominantly vegetative cells and low participation of sporulating forms.

Our results provided key information that contributes to better define the specific metabolic activity of each phenotype and the triggering factors inducing metabolic shifts. Particularly, the data presented in Chapter 4 suggest that clostridia phenotype are not responsible for solvent production, but a transition state preceding sporulation. This is in agreement with the first work dealing with FC application on the same strain (Tracy et al. 2008) and challenges the classical view of Clostridia phenotype as a butanol producer.

Although changes in membrane cell permeability due to phenotype evolution were observed by PI staining, a correlation between this parameter and butanol productivity or cell viability could not be found. Research on the application of different staining probes, together with flow cytometry complementary cell-sorting techniques would allow to further investigate the physiological heterogeneity in ABE extractive fermentation with solvents of different nature.

Some useful information about clostridia culture heterogeneity evolution during ABE fermentation set the basis for a new modeling approach. Considering the whole Clostridia cell cycle (acidogenic, solventogenic, sporulating forms) and the derived quantitative biomass heterogeneity evolution during ABE fermentation has been included in a mathematical model for the first time. Experimental data and existing knowledge were used to build the model and to define the main triggering factors inducing cell subpopulations transitions.

8.4 Process considerations: techno-economic outline and integration within a 2G-biorefinery

The industrialization of the ABE fermentation is being hampered by its production costs. In a general fermentation system, the main cost-associated process parameters are yield, product concentration and productivity, which correlates respectively with raw materials, OPEX and CAPEX costs. This thesis provides experimental input data to estimate the quantitative influence of the integrated process on these three key parameters. This shows that final product titer can be enhanced by extractive fermentation with solvents of different nature, thus leading to higher concentrated feedstock (lower water turnaround). Butanol yield might also be increased with specific polar solvents, like 2B1O. However, volumetric productivity was not improved with neither VO nor 2B1O. With the latter, productivity degradation was observed with scale up and can be attributed to enhanced biphasic toxicity.

Previous techno-economic studies of ABE fermentation coupled to ISPR technique focused on the energy savings in the recovery of the products from the aqueous broth (Oudshoorn et al. 2009, Qureshi et al. 2005), and only few works considered the specific energy demand of the separation technique implementation. Outram et al. (2016), compared the overall energy demand of OA-based extractive fermentation with control and alternative separation techniques. Nevertheless, solvents with different physic-chemical characteristics might lead to different process and regeneration schemes as seen in Chapter 6. However, none of previous works considered the impact of the physiological response of the microorganism towards the presence of the solvent. For instance, Luong inhibition function is classically used to predict productivity increase with toxicity alleviation. This thesis shows that productivity is not improved, even with high biocompatible solvents, suggesting that complex interphase solvent-microorganism interactions should not be ignored.

In this thesis, experimental data were available to account for actual fermentation performance parameters and for thermodynamic interactions between phases. Applying different solvents (2B1O versus VO) and operation modes (batch versus fed-batch), ABE extractive fermentation was comparatively assessed based on the main cost drivers: capital investment, feedstock, total plant energy requirements and wastewater treatment associated cost.

Combining Excel 2013 and ASPEN Plus v7.3, a butanol production extractive plant of 35 ktpa was simulated. Total energy demand decreased in all extractive configurations, and unexpectedly, VO-based fed-batch resulted in the greatest energy savings of 61%. Even though vegetable oil has a 10-times lower partition

coefficient respect to 2B1O, its regeneration cost was 4 MJ/kg BuOH, significantly lower than that reached with 2B1O (18 MJ/kg BuOH). However, the highest profit increase was achieved with 2B1O in fed-batch mode, lowering the minimum butanol selling price by 29% over conventional batch case. For this scenario, the economy was driven by more than 80% wastewater reduction, along with 34% savings in raw materials. Even if none of the simulated scenarios was profitable, a sensitivity analysis of the feedstock demonstrated that the relative profitability of the cases might be altered. It was found that below a sugar price cutoff of 200 €/t the integrated scheme could be competitive within the chemical industry. Moreover, the VO scenario becomes feasible at lower sugar prices.

In all studied configurations, substrate cost amounted up to minimum 60% of the overall cost of ABE fermentation, which agrees with previous works (Ranjan and Molhokar, 2012, Gapes,2001, Qureshi et al. 2005). The use of abundant and inexpensive substrates is essential from an economic point of view. Attention has been paid to lignocellulosic biomass as a 2G feedstock that do not compete with food market. Residues from agriculture and forestry may provide an interesting source for fermentation processes (Zhu et al. 2015). Moreover, a previous techno economical assessment of an organosolv based biorefinery (Sofia Laure et al. 2014) estimated that glucose production cost might reach 220 €/t, which is close to the critical required value for profitability calculated in this study. Pine, elm, reed, or beech wood have been subjected to organosolv pretreatment prior to ABE fermentation, with maximum ABE titers in the range of 11.6-15.1 g L-1 (Amiri and Karimi ,2015; Zhu et al. 2015; Tippkötter et al. 2014; Roth and Tippkötter 2016).

In Chapter 7, cellulose hydrolysate from organosolv pretreated beech wood was proved to be a suitable substrate to produce butanol by *C. beijerinckii* CECT 508 extractive fermentation. Comparative fermentations showed that the total solvent production increased by 40% in the hydrolysate medium compared to the control, and this was enhanced by 10% with extractive fermentation with 2B1O. Nevertheless, lag time in fermentations with hydrolysate was doubled, in agreement with Roth and Tippkötter (2016). The release of free phenols to the medium from the remaining lignin in cellulose fibers was suspected to be responsible for the lag time.

The use of laccases has been considered as a powerful and environmentally friendly tool to boost the conversion of pretreated lignocellulosic materials (Fillat et al., 2017). Laccase generates unstable phenoxy radicals from free phenols, inducing polymerization into less toxic high-molecular-mass products. Even if laccase has been successfully applied on pre-hydrolysate with significantly higher concentration of free phenols (Allard-Massicote et al. 2017), no information was available for the detoxification of organosolv hydrolysate prior to ABE

fermentation. Laccase treatment was evaluated on cellulose hydrolysates with and without remaining solids from the enzymatic hydrolysis. Unexpectedly, final ABE titer was enhanced by 25% in the medium with remaining solids. Moreover, in this case laccase treatment increased total solvent production in 24% with respect to the non-treated hydrolysate. In view to further scale up of the 2G-based biorefinery, the direct fermentation after enzymatic hydrolysis would alleviate investment cost and simplify the process scheme.

8.6Thesis implications and limitations

This thesis provides insights of the solvent-dependent phenomena associated to the ABE extractive fermentation process, and of their repercussion in the global scheme performance. This research enlarges the knowledge of the ABE fermentation in general, and particularly, reveals unattended responses of Clostridia in the presence of specific extracting solvents. Understanding the causes behind some of the observed phenomena, like the yield improvement in the presence of 2B1O, may help to exploit them and to improve future industrial process. On the other side, it has been pointed out that productivity degradation during scale up can be mostly attributed to increased biphasic toxicity. Thus, there are alternative bioreactor configurations where the application of highly capacitive solvents (like 2B1O) could be exploited. For example, the development of biofilm biphasic bioreactors, where most of cells are physically separated from the solvent interface, with potential additional protection conferred by the quorum sensum, should be investigated.

Particular attention was focused on the microbial population dynamics during ABE conventional and extractive fermentations. With an intrinsic complex cell cycle, Clostridia present different phenotypes and specific metabolic activities during batch fermentation. Consequently, despite of being a single-strain process, the culture is heterogeneous and formed by distinct subpopulations. Understanding the physiological factors that triggers the transition between the different cell types and monitoring the population dynamics is key to improve the knowledge of ABE fermentation and to innovate in further development of the process. For instance, our data challenged the classical view of Clostridia cell cycle, suggesting that acid and solvent producers are growth associated cells. VO based extractive fermentation is fully achieved with the participation of low sporulating forms, while with 2B1O the percentage of spores increased up to more than 40%, despite low aqueous butanol concentration (< 4 g/L). These results helped to build a mathematical model based governed by cell heterogeneity in batch bioreactor, which was able to fit own and literature data of ABE fermentation at free or fixed external pH.

The comparative techno-economic assessment of the integrated process reveals that its profitability is governed by the price of the feedstock. In this sense, 2B1O resulted to be more profitable since butanol yield increased more than 40%, in spite of the decreased productivity caused by toxicity. In extractive configurations, net energy demand decreased over control. However, when total final product concentration was increased the most impacting factor on OPEX economy was the reduction of WWT cost. Fed-batch mode allowed longer cycle operations and thus enhanced final product titer compared to batch operation. Instead, CAPEX is slightly impacted since volumetric capacity of the bioreactor must account for in situ solvent extraction. With free suspended cells, culture heterogeneity in the bioreactor has been reported to induce cyclic oscillations in product spectrum (Maddox et al. 1989, Li et al. 2011) which might difficult downstream. This might be partially faced by the implementation of biofilm bioreactor. Clostridium immobilization may lead not only to higher productivities but also to less output variation in a continuous bioreactor. In extractive configuration, continuous operation would imply to work at low dilution rates, in order to achieve full conversion of a highly concentrated feedstock.

Finally, another interesting approach to continue this work is the search of alternative and sustainable substrates for ABE extractive fermentation. The optimization of the integrated process within a 2G biorefinery might undoubtedly guide further developments.

8.7General conclusions

Most relevant conclusions are stated here.

On the solvent screening

- A trade off exists between extraction capacity (Kp) and selectivity (Sel) within the chemical families investigated. Inside the same family, branching degree is correlated with selectivity.
- A trade off exists between extraction capacity (Kp) and biocompatibility. LogP values between 4 and 6 limit a “critical region” toward *Clostridium acetobutylicum* behavior.

On the phenomena associated to ABE extractive fermentation:

- Interaction with specific solvents and ABE metabolism go beyond simple alleviation of inhibition by product removal.
- Butanol yield is enhanced with specific solvent presenting high affinity for butanol and for butyric acid (2B1O).

- Extractive fermentation with vegetable oil allows continuous butanol production with low sporulating forms participation.
- Extractive fermentation with 2B1O triggers sporulation sooner in the fermentation, even at butanol aqueous concentrations lower than 4 g/L.

On the integration of the ABE extractive process within an industrial biorefinery:

- Both extractive solvents lowered Minimum Butanol Selling Price, by a comparative techno-economic assessment.
- Yield governs production cost in all configurations evaluated.
- VO based solvents might be profitable at lower prices of feedstock.
- Organosolv hydrolysate was validated as a substrate for ABE extractive fermentation.

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List of Tables

Chapter 7

7.1 Laccase treatment of cellulose hydrolysate with and without S/L separation (HC and HCB respectively)

Chapter 8

8.1 Butanol results in extractive fermentation at different lab-scales

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

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