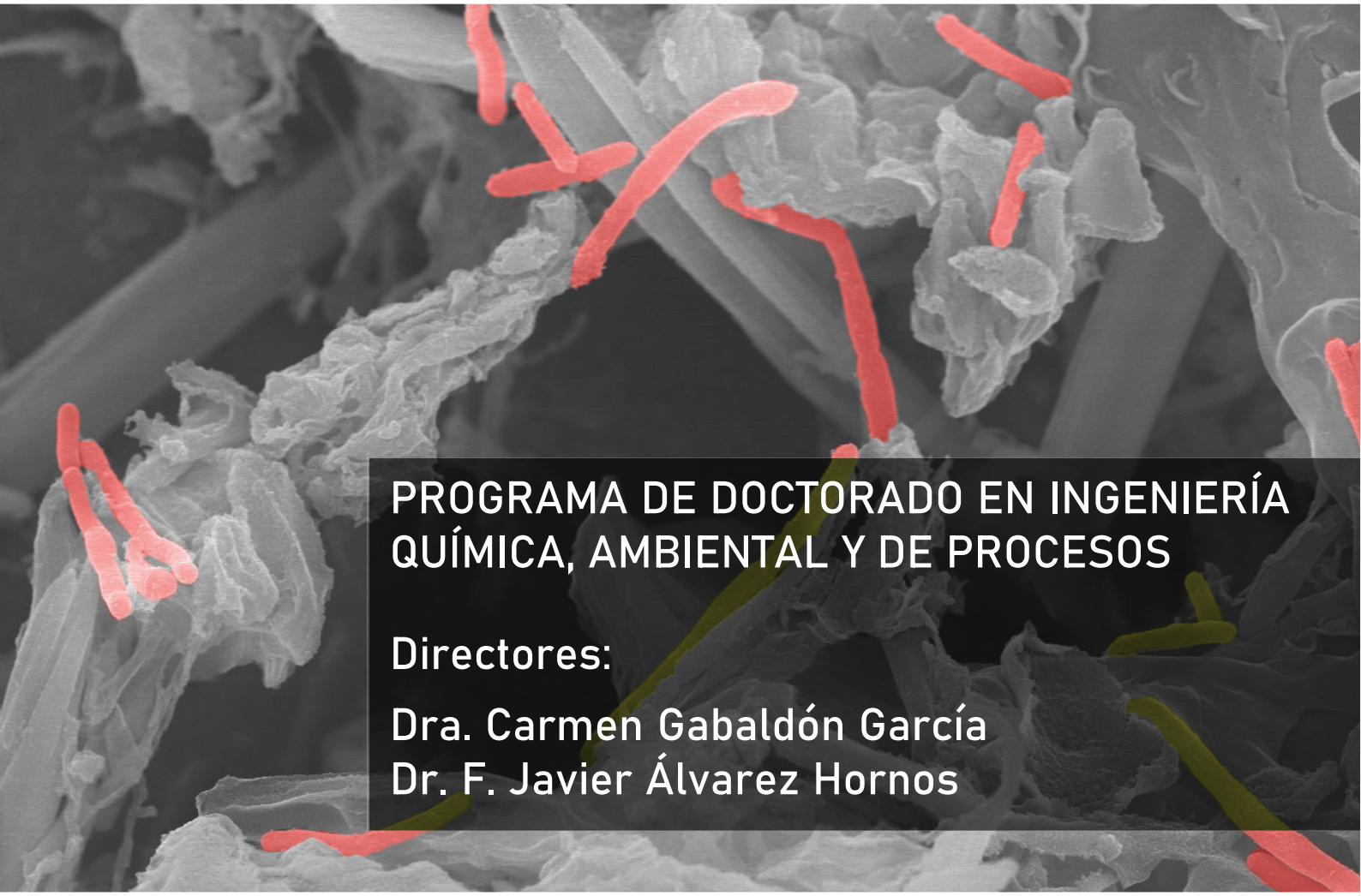
ESTUDIO DEL PROCESO SIMULTÁNEO DE SACARIFICACIÓN Y FERMENTACIÓN ACETO-BUTÍLICA-ETÍLICA A PARTIR DE PAJA DE ARROZ

Alejo Valles Sáez

Mayo 2022



PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA, AMBIENTAL Y DE PROCESOS

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VNIVERSITAT DE VALÈNCIA

Escola Tècnica Superior d'Enginyeria

Departament d'Enginyeria Química

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Programa de doctorado: Ingeniería Química, Ambiental y de Procesos

Memoria que, para optar al Título de Doctor por la Universitat de València, presenta **ALEJO VALLES SÁEZ**

Directores de tesis,
Dra. CARMEN GABALDÓN GARCÍA
Dr. F. JAVIER ÁLVAREZ HORNOS

Valencia, mayo de 2022

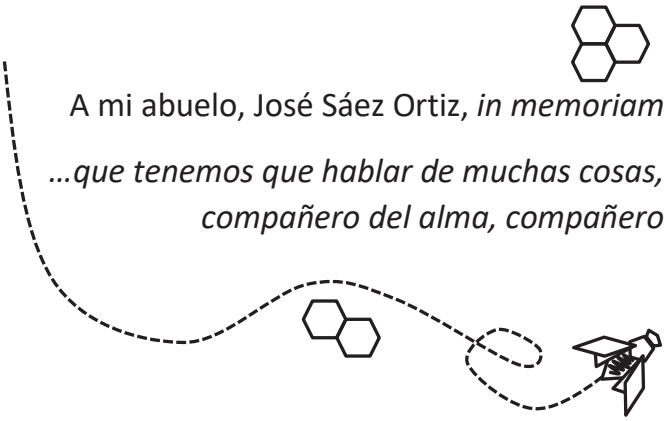
Dra. CARMEN GABALDÓN GARCÍA, Catedrática del Departamento de Ingeniería Química de la Universitat de València, y

Dr. F. JAVIER ÁLVAREZ HORNOS, Profesor Titular del Departamento de Ingeniería Química de la Universitat de València

CERTIFICAN: Que **Alejo Valles Sáez**, graduado en Biotecnología y con título de Máster en Biotecnología Industrial y Ambiental, ha realizado bajo su dirección el trabajo que bajo el título de: **"Estudio del proceso simultáneo de sacarificación y fermentación aceto-butílica-etílica a partir de paja de arroz"** presenta en esta Memoria y que constituye su Tesis para optar al Título de Doctor por la Universitat de València en el Programa de Doctorado en Ingeniería Química, Ambiental y de Procesos.

Y para que conste a los efectos oportunos firman el presente certificado en Valencia a mayo de 2022.

Fdo.: Dra. Carmen Gabaldón García Fdo.: Dr. F. Javier Álvarez Hornos



A mi abuelo, José Sáez Ortiz, *in memoriam*

…que tenemos que hablar de muchas cosas,
compañero del alma, compañero

Y sabed que yo estoy con vosotros todos los días hasta el fin del mundo

(Mt 28:20)

AGRADECIMIENTOS

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Agradezco que te podamos llamar Padre y Amigo. *Guíanos hasta el final de los tiempos*.

RESUMEN

El sistema energético de la Unión Europea está experimentando una profunda transformación con la finalidad de mitigar el cambio climático y de asegurar la independencia energética de sus Estados Miembro. Para ello se están llevando a cabo una serie de medidas como la implantación de una cuota mínima en el transporte del 3.5% de biocarburantes producidos a partir de residuos lignocelulósicos, entre otros biocombustibles, para 2030. En este contexto, toma relevancia la producción de biobutanol a partir de residuos lignocelulósicos mediante la fermentación aceto-butílica-etílica (ABE). Sin embargo, su implantación a nivel industrial continúa presentando una serie de desafíos entre los que se puede destacar la necesidad de múltiples etapas de proceso: pretratamiento, hidrólisis enzimática, fermentación y recuperación de producto. Una de las estrategias que permite reducir el número de etapas es el proceso de sacarificación y fermentación simultánea (SSF) que combina las etapas de hidrólisis enzimática y de fermentación en un mismo reactor.

El objetivo general de la presente tesis doctoral es el estudio del proceso SSF como una configuración alternativa al proceso convencional de sacarificación y fermentación separada (SHF) en la producción de biobutanol a partir de paja de arroz. Cabe destacar que, hasta la fecha, no se había evaluado el uso de este residuo en el proceso SSF. En esta tesis doctoral, se seleccionó *Clostridium beijerinckii* DSM 6422 como cepa bacteriana a emplear en la fermentación y se utilizó paja de arroz del Parque Natural de la Albufera (Valencia, España), icónico humedal del mediterráneo occidental en el que se requiere métodos de gestión de la paja que reduzcan el impacto de la producción de arroz sobre el medioambiente. La experimentación de la tesis doctoral se planificó mediante el diseño de experimentos como metodología estadística.

En los ensayos llevados a cabo para comparar los procesos SSF y SHF, se utilizó paja de arroz pretratada mediante hidrotermólisis asistida por microondas. Los resultados mostraron que la configuración simultánea incrementó en un 173% la productividad total de butanol en comparación con la configuración separada. Adicionalmente, se incluyó el análisis del efecto del pH inicial, de la concentración de extracto de levadura, de la concentración de hierro y de la carga enzimática sobre la producción de biobutanol en el proceso SSF, finalizando con una etapa posterior de optimización.

Los resultados del primer estudio pusieron de manifiesto la necesidad de mejorar la accesibilidad de la celulosa y la hemicelulosa de la paja de arroz a las enzimas hidrolíticas. Por este motivo se planificó un segundo estudio en el que se evaluó el pretratamiento alcalino con hidróxido sódico. Este estudio incluyó una aproximación novedosa en la que se utilizó como variable respuesta la relación butanol-biomasa, un parámetro que cuantifica la conversión de biomasa en biobutanol. Este parámetro permitió analizar el efecto de las variables del pretratamiento (temperatura y tiempo de reacción, concentración de hidróxido sódico y carga de sólidos) sobre la conversión de biomasa en biobutanol en el proceso SHF. La aplicación del pretratamiento con las condiciones optimizadas resultó en una conversión de biomasa en butanol de 78 g/kg junto con una producción de butanol de 10.1 g/L.

Finalmente, se desarrolló una configuración novedosa en la producción de biobutanol a partir de residuos lignocelulósicos que combinaba el proceso SSF con la recuperación *in situ* de producto (SSFR) y que operaba en modo de alimentación semicontinuo. En este estudio, se empleó paja de arroz sometida al pretratamiento alcalino optimizado. La aplicación del proceso SSFR en modo semicontinuo permitió duplicar la productividad de butanol (0.344 g/L h) en comparación con el proceso SSF en modo discontinuo (0.170 g/L h). Adicionalmente, se evaluó la posibilidad de reducir la carga enzimática y la concentración de los componentes del medio en esta configuración avanzada.

RESUM

El sistema energètic de la Unió Europea està experimentant una profunda transformació amb la finalitat de mitigar el canvi climàtic i d'assegurar la independència energètica dels seus Estats Membres. Per això s'estàn duent a terme una sèrie de mesures com la implantació d'una quota mínima en el transport del 3.5% de biocarburants produïts a partir de residus lignocel·lulòsics, entre altres biocombustibles, per a 2030. En aquest context, pren rellevància la producció de biobutanol a partir de residus lignocel·lulòsics mitjançant la fermentació aceto-butílica-etílica (ABE). No obstant això, la seua implantació a nivell industrial continua presentant una sèrie de desafiaments entre els quals es pot destacar la necessitat de múltiples etapes de procés: pretractament, hidròlisi enzimàtica, fermentació i recuperació de producte. Una de les estratègies que permet reduir el nombre d'etapes és el procés de sacarificació i fermentació simultània (SSF) que combina les etapes d'hidròlisi enzimàtica i de fermentació en un mateix reactor.

L'objectiu general de la present tesi doctoral és l'estudi del procés SSF com una configuració alternativa al procés convencional de sacarificació i fermentació separada (SHF) en la producció de biobutanol a partir de palla d'arròs. Cal destacar que, fins ara, no s'havia avaluat l'ús d'aquest residu en el procés SSF. En aquesta tesi doctoral, es va seleccionar *Clostridium beijerinckii* DSM 6422 com a soca bacteriana a emprar en la fermentació i es va utilitzar palla d'arròs del Parc Natural de l'Albufera (València, Espanya), icònic aiguamoll del mediterrani occidental on cal mètodes de gestió de la palla que reduïsquen l'impacte de la producció d'arròs sobre el medi ambient. L'experimentació de la tesi doctoral es va planificar mitjançant el disseny d'experiments com a metodologia estadística.

En els assaigs duts a terme per a comparar els processos SSF i SHF, es va utilitzar palla d'arròs pretractada mitjançant hidrotermòlisi assistida per microones. Els resultats van mostrar que la configuració simultània va incrementar en un 173% la productivitat total de butanol en comparació amb la configuració separada. Addicionalment, es va incloure l'anàlisi del efecte del pH inicial, de la concentració d'extracte de llevat, de la concentració de ferro i de la càrrega enzimàtica sobre la producció de biobutanol en el procés SSF, finalitzant amb una etapa posterior d'optimització.

Els resultats del primer estudi van posar de manifest la necessitat de millorar l'accessibilitat de la cel·lulosa i la hemicel·lulosa de la palla d'arròs als enzims hidrolítics. Per aquest motiu es va planificar un segon estudi en el qual es va avaluar

el pretractament alcalí amb hidròxid sòdic. Aquest estudi va incloure una aproximació innovadora què va utilitzar com a variable resposta la relació butanol-biomassa, un paràmetre que quantifica la conversió de biomassa en biobutanol. Aquest paràmetre va permetre analitzar l'efecte de les variables del pretractament (temperatura i temps de reacció, concentració d'hidròxid sòdic i càrrega de sòlids) sobre la conversió de biomassa en biobutanol en el procés SHF. L'aplicació del pretractament amb les condicions optimitzades va resultar en una conversió de biomassa en butanol de 78 g/kg juntament amb una producció de butanol de 10.1 g/L.

Finalment, es va desenvolupar una configuració innovadora en la producció de biobutanol a partir de residus lignocel·lulòsics que combinava el procés SSF amb la recuperació *in situ* de producte (SSFR) i que operava amb alimentació semicontínua. En aquest estudi, es va emprar palla d'arròs sotmesa al pretractament alcalí optimitzat. L'aplicació del procés SSFR en operació semicontínua va permetre duplicar la productivitat de butanol (0.344 g/L h) en comparació amb el procés SSF en operació discontínua (0.170 g/L h). Addicionalment, es va avaluar la possibilitat de reduir la càrrega enzimàtica i la concentració dels components del medi en aquesta configuració avançada.

SUMMARY

The European Union's energy system is going through a profound transformation in an attempt to mitigate the effects of climate change and ensure that the member states retain their energy independence. This involves a number of changes, including imposing a minimum quota of 3.5% of lignocellulosic-based biofuels, among other biofuels, for transport by 2030. In this context lignocellulosic-based biobutanol by acetone-butanol-ethanol (ABE) fermentation is gaining importance. However, its production on an industrial scale still faces some challenges, one of the most important being the many stages the process requires: pretreatment, enzymatic hydrolysis, fermentation and product recovery. Simultaneous saccharification and fermentation (SSF), which combines enzymatic hydrolysis and fermentation in a single reactor, is one of the strategies that can be used to reduce the number of stages.

The general aim of the present doctoral thesis is to study SSF as an alternative configuration to the conventional separate saccharification and fermentation processes (SHF) to produce biobutanol from rice straw. It should be noted that to date the use of this residue has not been considered for SSF. For this thesis *Clostridium beijerinckii* DSM 6422 was selected as the bacterial fermentation strain and it was used rice straw from the *Parque Natural de la Albufera* (Valencia, Spain), iconic Western Mediterranean wetlands where management methods for straw are necessary to reduce the impact of rice production on the environment. The experiments involved in the thesis were planned by statistical design of experiments.

Rice straw pretreated by microwave-assisted hydrothermal hydrolysis was used in the tests carried out to compare SSF and SHF. The results showed that the simultaneous configuration increased total butanol productivity over the separate configuration by 173%. The effect of initial pH, yeast extract concentration, iron concentration and enzyme loading on biobutanol production was also analysed in the SSF process, with a final optimisation stage.

As the results of the first study showed that there was a need to improve rice straw cellulose and hemicellulose accessibility to hydrolytic enzymes, a second study was planned to evaluate alkali pretreatment with sodium hydroxide, including a novel approach using as a variable response the butanol-biomass ratio, the parameter that quantifies biomass conversion to biobutanol. This parameter allowed to analyze the effect of the pretreatment variables (temperature and

reaction time, sodium hydroxide concentration and solid loafing) on biomass conversion to biobutanol in SHF. Applying the pretreatment in the optimal conditions resulted in a biomass conversion to biobutanol of 78 g kg^{-1} together with a butanol production of 10.1 g L^{-1}.

Finally, a novel lignocellulosic-based biobutanol production configuration was created combining SSF with *in situ* product recovery (SSFR) operating in fed-batch mode. In this study, it was used rice straw pretreated with optimal conditions derived from the second study. Applying fed-batch SSFR doubled butanol productivity (0.344 g L^{-1} h^{-1}) over SSF (0.170 g L^{-1} h^{-1}). The possibility of reducing the enzyme loading and the concentration of medium components was also evaluated in this advanced configuration.

ÍNDICE

1. ESTRUCTURA DE LA TESIS

Esta tesis doctoral consta de 12 capítulos y se presenta como compendio de 3 artículos científicos que constituyen los capítulos 6, 7 y 8 como una adaptación de los manuscritos originales ya publicados. Las publicaciones originales y el material suplementario se muestran en el anexo de este documento (capítulo 12). Tras exponer la estructura de la tesis en el presente capítulo, se presentan dos capítulos introductorios relativos a los biocarburantes de segunda generación (capítulo 2) y a la producción de biobutanol a partir de residuos lignocelulósicos mediante fermentación ABE (capítulo 3), incluyendo una revisión bibliográfica del estado del arte actual. El capítulo 4 describe el objetivo general y los objetivos parciales y específicos de la tesis y el capítulo 5 presenta un resumen de los materiales y métodos utilizados junto con el plan de trabajo. Finalmente, este documento incluye las conclusiones obtenidas en la investigación junto con las perspectivas de futuro (capítulo 9), el resumen extendido de la tesis (capítulo 10) y los acrónimos empleados (capítulo 11). Las referencias completas de los artículos científicos son las siguientes:

- Artículo I (capítulo 6): Valles, A., Álvarez-Hornos, F.J., Martínez-Soria, V., Marzal, P., Gabaldón, C., 2020. Comparison of simultaneous saccharification and fermentation and separate hydrolysis and fermentation processes for butanol production from rice straw. Fuel 282, 118831. https://doi.org/10.1016/j.fuel.2020.118831.
- Artículo II (capítulo 7): Valles, A., Capilla, M., Álvarez-Hornos, F.J., García-Puchol, M., San-Valero, P., Gabaldón, C., 2021. Optimization of alkali pretreatment to enhance rice straw conversion to butanol. Biomass and Bioenergy 150, 106131. https://doi.org/10.1016/j.biombioe.2021.106131.
- Artículo III (capítulo 8): Valles, A., Álvarez-Hornos, F.J., Capilla, M., San-Valero, P., Gabaldón, C., 2021. Fed-batch simultaneous saccharification and fermentation including *in-situ* recovery for enhanced butanol production from rice straw. Bioresource Technology 342, 126020. https://doi.org/10.1016/j.biortech.2021.126020.

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2. BIOCARBURANTES DE SEGUNDA GENERACIÓN

2.1. Biocarburantes como factor de transformación del sector energético

La Convención Marco de las Naciones Unidas sobre el Cambio Climático (*United Nations Framework Convention on Climate Change*, UNFCCC), adoptada el 9 de mayo de 1992 ante los efectos adversos de los cambios del clima, definió a los gases de efecto invernadero (*Greenhouse Gases*, GHG) como: *"aquellos componentes gaseosos de la atmósfera, tanto naturales como antropógenos, que absorben y reemiten radiación infrarroja"* (artículo 1, punto 5).

Desde 1990 los seres humanos hemos incrementado en un 50% las emisiones de gases que contribuyen directamente al efecto invernadero según los datos aportados por el Instituto de Recursos Mundiales (*World Resources Institute*, WRI; WRI, 2021), emitiéndose 48940 millones de toneladas equivalentes de dióxido de carbono en 2018. El principal contaminante fue el dióxido de carbono (75%) seguido por el metano (17%), el óxido nitroso (6%) y los gases fluorados (2%). En la Figura 2.1 se muestran los porcentajes de GHG emitidos en el año 2018 por sector, donde se observa que la principal fuente de emisión de estos gases es la obtención de energía con una contribución del 76%. El sector energético incluye la generación de electricidad y calor, el transporte y la fabricación y construcción.

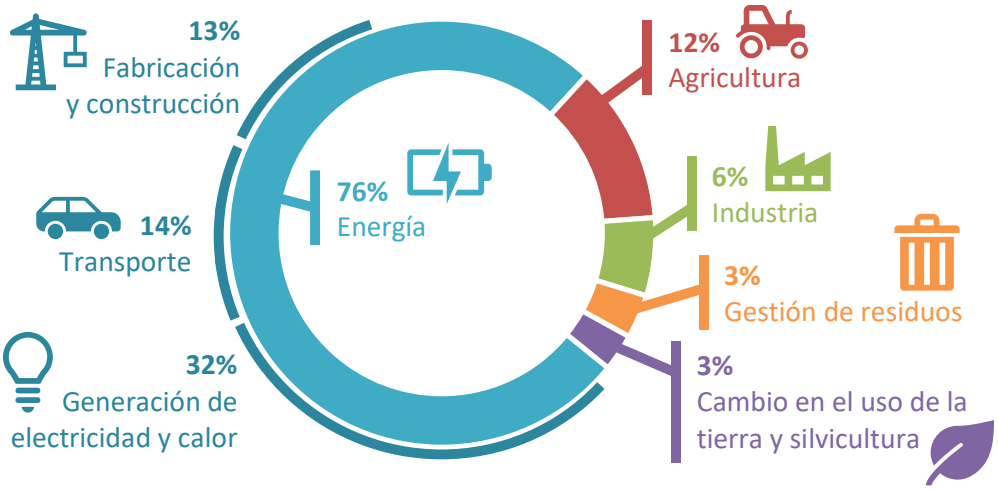

Figura 2.1. Emisiones antropogénicas globales de GHG por sector en 2018. Elaboración propia a partir de datos facilitados por WRI (2021).

El 93% del dióxido de carbono liberado a la atmósfera procede de la quema de carbón, gas natural y petróleo como combustibles fósiles (WRI, 2021). Esta actividad es característica del sector energético, especialmente del transporte donde se consume el 66% de los productos derivados del petróleo y el 7% del gas natural (IEA, 2019a). A pesar del fuerte descenso en la demanda del petróleo debido a la emergencia sanitaria del COVID-19, la Agencia Internacional de la Energía

(*International Energy Agency*, IEA) prevé que en 2023 se recuperen los valores de 2019 con una demanda de 101.2 millones de barriles de petróleo al día (IEA, 2021).

Las emisiones de GHG han aumentado la temperatura del planeta, siendo esta la principal causa del cambio climático, definido por la UNFCCC como *"un cambio de clima atribuido directa o indirectamente a la actividad humana que altera la composición de la atmósfera mundial y que se suma a la variabilidad natural del clima observada durante períodos de tiempo comparables"* (artículo 1, punto 2).

De acuerdo con un informe publicado en agosto de 2019 por el Grupo Intergubernamental de Expertos sobre el Cambio Climático (*Intergovernmental Panel on Climate Change*, IPCC), la temperatura del aire en la superficie terrestre ha aumentado entre 1.38 y 1.68 °C desde el periodo preindustrial (1850 – 1900). Una de las consecuencias de este suceso es el incremento de la frecuencia e intensidad en los últimos años de fenómenos extremos como olas de calor, sequías, inundaciones, incendios forestales y riadas (IPCC, 2019a). Por otra parte, la extensión de la criosfera se ha visto reducida y los océanos han experimentado un aumento de la temperatura, un incremento de la acidificación y de la estratificación y una pérdida de oxígeno. La velocidad de subida del nivel medio del mar alcanzó los 3.6 mm/año debido, principalmente, a la pérdida de los mantos de hielo y de los glaciales (IPCC, 2019b). Todo ello supone un elevado riesgo para la salud humana y de los ecosistemas, la seguridad alimentaria, la infraestructura, el transporte, la economía y la política. De hecho, la Organización Mundial de la Salud (*World Health Organization*, WHO) estimó que en el periodo comprendido entre 2030 y 2050 morirán cada año 250000 personas a causa del cambio climático, entre las cuales 95000 serán por desnutrición infantil, 60000 por paludismo, 48000 por diarrea y 38000 por golpes de calor (WHO, 2014).

En este contexto, la producción y consumo de biocarburantes, definidos como *"los combustibles líquidos destinados al transporte y producidos a partir de biomasa"* (artículo 2, punto 33; Unión Europea: Parlamento Europeo y Consejo, 2018), permite reducir notablemente las emisiones de GHG. Otros beneficios derivados de la generación y uso de biocarburantes en una determinada región son el fortalecimiento de su sector agrícola y el fomento de su autosuficiencia energética. Los biocarburantes con mayor potencial para reemplazar a los combustibles convencionales son el biobutanol, el bioetanol, el bio-ETBE (*ethyl-tert-butyl-ether*), el biometanol, el bio-MTBE (*methyl-tert-butyl-ether*), el gasóleo verde, la gasolina verde y el biodiesel (Puricelli y col., 2021). En 2019 la producción global de biocarburantes alcanzó los 162 billones de litros, de los cuales el 71% se

corresponden a bioetanol, obtenido mayoritariamente en Estados Unidos y Brasil, y el 25% a biodiesel, obtenido mayoritariamente en la Unión Europea (IEA, 2020).

Los biocarburantes se clasifican en función del tipo de sustrato utilizado para producirlos. Mientras que los biocarburantes de primera generación o convencionales son aquellos derivados de cultivos alimenticios y forrajeros, los de segunda generación son aquellos derivados de cultivos energéticos, residuos agrícolas, residuos forestales, residuos sólidos urbanos y otros desechos como grasas animales y vegetales. Las principales ventajas de los biocarburantes de segunda generación son que su obtención no afecta a la seguridad alimentaria ni al precio de los alimentos y que tienen un mayor potencial referente a la reducción de GHG que los de primera generación siempre que no impliquen el cambio en el uso de la tierra. Sin embargo, se encuentran en proceso de desarrollo y su producción es más compleja y presenta, en la actualidad, mayores costes de operación. Por otra parte, el biodiesel producido a partir de las microalgas es denominado como biocarburante de tercera generación. No obstante, en el estado actual del proceso, la utilización de microalgas para producir biodiesel genera una emisión de GHG mayor a la que generan los combustibles fósiles (Jeswani y col., 2020).

Actualmente, los sustratos más usados en la producción de bioetanol son el maíz, la mandioca y la caña de azúcar, mientras que la soja y el aceite de palma lo son en la producción de biodiesel. De este modo, la mayor parte de biomasa utilizada como sustrato en la obtención de los biocarburantes con mayor producción global procede de cultivos alimenticios, mientras que el porcentaje de biocombustibles obtenidos a partir de residuos es del 9% (IEA, 2019b).

La Unión Europea aprobó el 11 de diciembre de 2018 la Directiva 2018/2001 relativa al fomento del uso de energía procedente de fuentes renovables (Unión Europea: Parlamento Europeo y Consejo, 2018). Esta directiva, que está en consonancia con el Acuerdo de Paris de 2015 sobre el cambio climático, procede de la refundición de la Directiva 2009/28/CE del 23 de abril de 2009 relativa al fomento del uso de energía procedente de fuentes renovables y por la que se modifican y se derogan las Directivas 2001/77/CE y 2003/30/CE (Unión Europea: Parlamento Europeo y Consejo, 2009). Entre sus objetivos vinculantes para 2030 se encuentran la reducción de las emisiones de GHG al menos en un 40% con respecto a 1990 y la consecución de una cuota de energías renovables de al menos un 32%. El porcentaje requerido de energías renovables se fijó específicamente para el sector del transporte en un 14% y, para lograrlo, se han impuesto una serie de obligaciones a los Estados Miembro como la de garantizar una cuota mínima del 3.5% de biocarburantes y biogás producidos a partir de determinadas materias primas

referente al consumo final de energía en el sector del transporte. Entre las materias primas enumeradas en la parte A del anexo IX de esta directiva se encuentran diversos residuos lignocelulósicos, fangos de depuradoras, desechos urbanos y biomasa de algas, pero se descarta la biomasa agrícola obtenida en turberas, tierras de elevado valor y tierras ricas en reservas de carbono. La directiva resalta la importancia de limitar también el uso de biocarburantes producidos a partir de cultivos destinados a la alimentación humana o animal, ya que el cambio indirecto en el uso de la tierra contribuye a aumentar las emisiones de GHG.

Aparte de este marco regulador común, otras acciones están transformando el sistema energético europeo como la firma del Pacto Verde Europeo el 11 de diciembre de 2019 (Unión Europea: Comisión Europea, 2019). Este pacto aspira alcanzar la neutralidad climática en 2050 mediante medidas como la eliminación de las subvenciones a los combustibles fósiles y el fomento de la producción y uso de combustibles alternativos. Tal y como se indica en la Directiva 2014/94/CE del 22 de octubre de 2014 (Unión Europea: Parlamento Europeo y Consejo, 2014), los combustibles alternativos más importantes son los biocarburantes.

En 2019 la cuota de energías renovables en la Unión Europea fue del 19.7%, lo que indica una correcta transformación del sistema energético como resultado del esfuerzo realizado por parte de los Estados Miembro. Dentro del sector del transporte este porcentaje fue del 8.9% (EUROSTAT, 2021). No obstante, la cuota de consumo de biocombustibles líquidos, biogás y biometano alcanza en la actualidad únicamente el 3.5% del total de combustibles y gases. En dirección opuesta a las directrices marcadas, prácticamente la totalidad de estos combustibles renovables se obtiene a partir de cultivos alimenticios y forrajeros, por lo que su producción y consumo está limitado por la inseguridad jurídica (Unión Europea: Comisión Europea, 2020).

2.2. Biocarburantes de segunda generación: vías de obtención

Los biocarburantes de segunda generación más estudiados son el biometanol, el bioetanol, el biobutanol, el gasóleo verde y el biodiesel (Fivga y col., 2019). A modo de resumen, en la Figura 2.2 se muestran las principales vías por las que se obtienen dichos biocarburantes junto con el contenido energético por volumen de cada compuesto. La elección de las diferentes tecnologías termoquímicas (gasificación e hidrotratamiento) y bioquímicas (transesterificación y fermentación) depende tanto del tipo de residuo disponible como de la demanda del producto en el mercado (Fivga y col., 2019). En referencia al contenido

energético por volumen, estos biocarburantes presentan un potencial comprendido entre 16 y 34 MJ/L.

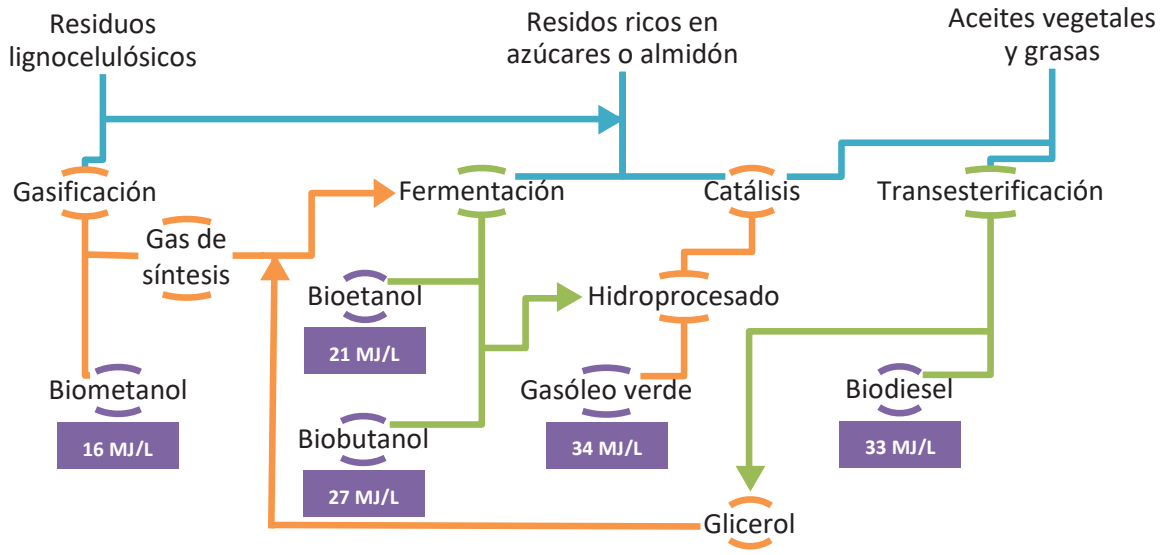

Figura 2.2. Principales vías de obtención de los biocarburantes de segunda generación. Adaptada de Fivga y col. (2019).

En la gasificación, los residuos lignocelulósicos reaccionan durante 3 – 4 segundos con aire, oxígeno o vapor a una temperatura ente 600 y 1000 °C, produciéndose metano, nitrógeno, hidrógeno, dióxido de carbono, monóxido de carbono e hidrocarbonos. Posteriormente, a partir del gas crudo se obtiene el gas de síntesis, formado por cantidades variables de dióxido de carbono e hidrógeno (Ibarra-Gonzalez y Rong, 2019). Este proceso presenta, frente a la fermentación de los residuos lignocelulósicos, la ventaja potencial de aprovechar la lignina. El hidrotratamiento es otro proceso termoquímico en el que se genera gasóleo verde al reaccionar con hidrógeno los alcoholes obtenidos en la fermentación, los productos de catálisis de residuos ricos en azúcares o almidón y los productos de catálisis con zeolita o hidrodesoxigenación de aceites y grasas. Otras vías termoquímicas son la pirólisis y el proceso Fischer-Tropsch. En comparación con las tecnologías termoquímicas, las tecnologías bioquímicas presentan temperaturas y presiones de operación más bajas. En la transesterificación, los triglicéridos y ácidos grasos libres, procedentes de aceites vegetales y animales, forman ésteres metílicos de ácidos grasos conocidos como biodiesel FAME (*Fatty Acid Methyl Ester*), glicerol y agua al reaccionar con metanol en presencia de un catalizador alcalino como hidróxido sódico o hidróxido potásico. Otra alternativa bioquímica es la fermentación de gas de síntesis, de sustratos ricos en azúcares o de glicerol por parte de levaduras o bacterias para producir bioetanol o biobutanol (Fivga y col., 2019).

Actualmente, los biocarburantes de segunda generación con mayor producción a nivel mundial son el gasóleo verde y el biodiesel derivados de aceites vegetales y grasas. Por el contrario, la producción de biocarburantes a partir de residuos ricos en lignocelulosa, azúcar o almidón es considerablemente menor y se mantiene en gran parte restringida a plantas de demostración de pequeña escala debido al limitado desarrollo de las tecnologías de obtención. Sin embargo, el interés en las vías termoquímicas y fermentativas ha aumentado recientemente, ya que los residuos agrícolas y forestales son un tipo de biomasa muy abundante y barata. De esta forma, la mayoría de nuevas instalaciones relativas a este campo están destinadas a la transformación a gran escala de residuos lignocelulósicos como bagazo, rastrojo de maíz y fibra de maíz en bioetanol (IEA, 2019b).

Una característica importante de los biocarburantes para su uso es el grado en el que se pueden mezclar con los combustibles convencionales y utilizarse en los motores existentes. La mezcla es función de las propiedades de los compuestos como, por ejemplo, el octanaje, el contenido en agua, la viscosidad y el punto de inflamabilidad, pero está limitada por la regulación específica de cada territorio. En Europa, el bioetanol puede añadirse a la gasolina hasta un límite del 10% (v/v, mezcla E10) según el estándar EN 15376, mientras que en Estados Unidos se puede llegar hasta el 15% (v/v, mezcla E15) según el estándar ASTM D4806. El biodiesel FAME, regulado por los estándares EN 14214 y ASTM D6751, puede añadirse al gasóleo hasta un límite del 7 – 10% (v/v, mezcla B7 – B10). Una de las ventajas del gasóleo verde y del biobutanol es que ambos biocarburantes pueden usarse directamente en motores convencionales (Fivga y col., 2019; Puricelli y col., 2021). El porcentaje de biobutanol permitido en gasolina varía entre 1 y 12.5% (v/v) en Estados Unidos de acuerdo al estándar ASTM D7862 (ASTM, 2021). Por otra parte, se ha demostrado que el biobutanol cumple los estándares europeos EN 228 y EN 15376 al incluirse en gasolina o en la mezcla E85 (Lapuerta y col., 2017). En referencia al biometanol, la Unión Europea permite un porcentaje de mezcla con gasolina (3% p/v) muy pequeño (Schubert, 2020).

2.2.1. Biocarburantes de segunda generación de origen fermentativo

Los principales biocarburantes de segunda generación obtenidos en vías fermentativas son el bioetanol (C_2H_6O) y el biobutanol ($C_4H_{10}O$), alcoholes considerados como alternativas prometedoras a la gasolina. El término “biobutanol” hace referencia a uno de los cuatro isómeros del butanol, el butanol normal (n-butanol o 1-butanol), obtenido a partir de biomasa. Los otros tres isómeros son el butanol secundario (s-butanol o 2-butanol), el isobutanol (i-butanol)

y el butanol terciario (terbutanol), presentando distintas propiedades en función del tipo de cadena de carbonos y la posición del grupo hidroxilo (Veza y col., 2021).

La comparación de las propiedades fisicoquímicas del etanol, el butanol y la gasolina se muestra en la Tabla 2.1. Los puntos de inflamabilidad del etanol y el butanol son, respectivamente, 13 y 35 °C, por lo que su manejo y transporte es seguro. La manipulación del butanol es especialmente estable debido al alto grado de higroscopicidad que le otorga su elevado peso molecular (74.12 g/mol). Un peso molecular alto también se traduce en una baja volatilidad y en una alta viscosidad. A pesar de que el etanol presenta mayor octanaje (98 – 129) que el butanol (96), tiene una alta temperatura de ignición (425 °C), mientras que la del butanol es menor (397 °C). Otras de las ventajas que ofrece el butanol como carburante es su elevado cociente aire-combustible (11), su elevado contenido energético por volumen (27 – 30 MJ/L) y que su uso conlleva menos problemas de corrosión que en el caso del etanol.

Tabla 2.1. Propiedades fisicoquímicas del etanol, el butanol y la gasolina como carburantes. Datos obtenidos de Dürre (2007), Fivga y col. (2019) y Schubert (2020).

Propiedad	Etanol	Butanol	Gasolina
Punto de inflamabilidad (°C)	13	35	-65--45
Peso molecular (g/mol)	46.07	74.12	100-105
Octanaje	98-129	96	86-99
Temperatura de ignición (°C)	425	397	257
Cociente aire-combustible	3	11	15
Densidad energética (MJ/L)	21	27-30	32

Los microorganismos que llevan a cabo las fermentaciones para la obtención de bioetanol o biobutanol de segunda generación pueden metabolizar cuatro tipos de sustrato: el gas de síntesis, el glicerol, los residuos ricos en azúcares o almidón y los residuos lignocelulósicos (Figura 2.2). El gas de síntesis procede de diversos orígenes como la gasificación de biomasa residual y la captura de dióxido de carbono acoplada a la electrólisis de agua y su utilización no requiere etapas adicionales. Algunas bacterias autotróficas y acetogénicas del género *Clostridium*, como *C. ljungdahlii* y *C. autoethanogenum*, son capaces de asimilar anaeróbicamente el gas de síntesis mediante las vías acetil-CoA o Wood-Ljungdahl y producir bioetanol. La vía Wood-Ljungdahl también es utilizada por especies bacterianas *Clostridium* y *Butyribacterium* para producir biobutanol a partir de gas de síntesis. Uno de los retos que dificultan este tipo de producción de alcoholes son las limitaciones asociadas a la transferencia de materia (Schubert, 2020). El glicerol

crudo como producto de la síntesis de biodiesel contiene impurezas como metanol, sales y ácidos grasos que inhiben el crecimiento celular, por lo que su utilización suele requerir una etapa de purificación (Kaushal y col., 2019). En la fermentación de glicerol se suelen seleccionar bacterias como *Escherichia coli*, *Klebsiella planticola* y *Enterobacter aerogenes* para obtener bioetanol y *C. pasteurianum*, *K. pneumoniae* y *E. coli* para obtener biobutanol. Muchas de estas especies bacterianas han sido modificadas genéticamente para producir estos disolventes a partir de glicerol (Schubert, 2020).

La asimilación de residuos ricos en azúcares o almidón requiere una etapa previa de hidrólisis y, además, los residuos lignocelulósicos han de someterse a un pretratamiento al tratarse de un material recalcitrante. Estas dos etapas adicionales incrementan notablemente los costes y han de considerarse en la evaluación técnico-económica del proceso. El análisis económico de la obtención de bioetanol y biobutanol a partir de residuos lignocelulósicos varía ampliamente como consecuencia de los cambios en la composición de la biomasa, entre otros motivos. Mientras que el coste de producción estimado para el bioetanol es 0.4 – 13.7 €/kg, el del biobutanol es 0.5 – 2.3 €/kg. En referencia a la producción de bioetanol a partir de azúcares, las levaduras pertenecientes al género *Saccharomyces* y *Candida*, como *S. uvarum*, *S. cerevisiae* y *C. utilis*, consumen eficazmente hexosas en condiciones anaeróbicas. Por el contrario, pocas cepas son capaces de consumir pentosas y las metabolizan con una baja productividad. *S. cerevisiae* presenta un alto grado de tolerancia a inhibidores procedentes del pretratamiento de la biomasa lignocelulósica y al etanol. La producción de biobutanol a partir de azúcares se lleva a cabo mayoritariamente mediante fermentación ABE (*acetone-butanol-ethanol*) por bacterias pertenecientes al género *Clostridium*, como *C. acetobutylicum* y *C. beijerinckii*, capaces de asimilar tanto hexosas como pentosas (Schubert, 2020).

2.2.2. Producción de biocarburantes de origen fermentativo a partir de residuos lignocelulósicos: etapas de proceso

Tal y como se ha comentado, la producción fermentativa de biocarburantes a partir de residuos lignocelulósicos necesita dos etapas previas, pretratamiento e hidrólisis, que permiten la liberación de azúcares fermentables de la biomasa. Estas etapas son necesarias, debido a que la gran mayoría de levaduras y bacterias no pueden metabolizar directamente la celulosa ni la hemicelulosa (Sánchez y Montoya, 2013).

La biomasa lignocelulósica es un tipo de material vegetal compuesto mayoritariamente por celulosa, hemicelulosa y lignina, tal y como ilustra la Figura

2.3. Estos materiales también incluyen, en menor medida, pectina, extractivos, cenizas y proteínas. El principal componente de la biomasa lignocelulósica es la celulosa, un polímero lineal de hasta 15000 unidades de β-D-glucopiranosa unidas por enlaces glucosídicos β –(1.4). Entre 20 y 300 cadenas de celulosa se unen mediante puentes de hidrógeno y fuerzas de van der Waals para formar una microfibrilla de celulosa. Las microfibrillas, a su vez, se agregan de forma ordenada y desordenada dando lugar, respectivamente, a partes cristalinas y amorfas que se alternan e insertan en una matriz de hemicelulosa. La unión entre celulosa y hemicelulosa se lleva a cabo por enlaces covalentes y puentes de hidrógeno. La hemicelulosa contiene tanto pentosas (D-xilosa, L-ramnosa y L-arabinosa) como hexosas (D-glucosa, D-galactosa y D-manosa), las cuales forman cadenas cortas y ramificadas a partir de enlaces glucosídicos β –(1.4) y β –(1.3). Estas cadenas cortas y ramificadas son fácilmente degradables en comparación con las largas cadenas de la celulosa. La hemicelulosa también contiene ácido 4-O-metilglucurónico, D-glucurónico y D-galacturónico y puede incluir grupos acetilo. El complejo celulosa-hemicelulosa está recubierto por la lignina, un polímero fenólico de alcohol coniferil, cumaril y sinapil. En el material vegetal este polímero hidrofóbico se encuentra en la pared celular donde es el responsable de otorgar rigidez, minimizar el estrés oxidativo y evitar la entrada de microorganismos (Bajpai, 2016a).

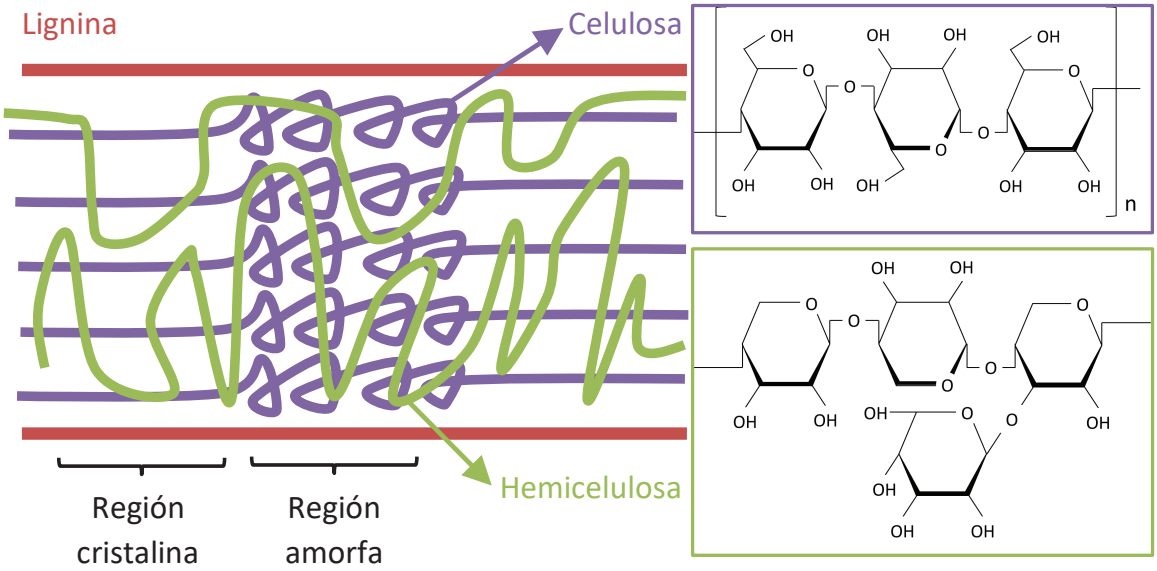

Figura 2.3. Esquema de la biomasa lignocelulósica y estructura química de la celulosa y hemicelulosa. Adaptada de Bajpai (2016a).

La naturaleza recalcitrante de los residuos lignocelulósicos se basa en una serie de factores químicos como el grado de polimerización de la celulosa y la hemicelulosa, la presencia de grupos acetilo e hidroxilo, el contenido en lignina y hemicelulosa y las interacciones entre los tres polímeros, así como en factores

físicos como la cristalinidad, el área superficial y el volumen accesible (Zoghlami y Paës, 2019). La lignina actúa como un obstáculo físico frente a la acción de las enzimas hidrolíticas, las adsorbe de forma no específica e impide la formación del complejo enzima-carbohidrato (Bajpai, 2016a). La finalidad específica del pretratamiento es la de facilitar la accesibilidad a la celulosa y la hemicelulosa en la hidrólisis posterior y/o la degradabilidad de ambos polímeros. Para ello, mediante la rotura de la matriz que une los tres componentes principales (celulosa, hemicelulosa y lignina) y la deslignificación, referente a la eliminación de lignina, se altera el complejo lignina-hemicelulosa, se disminuye la cristalinidad de la celulosa y el grado de polimerización y se aumenta la porosidad del material (Kim, 2018; Putro y col., 2016). Se ha estimado que el pretratamiento de un residuo lignocelulósico como el rastrojo de maíz mediante ácido sulfúrico diluido supone el 23 y el 11% de los costes totales de producción a gran escala de bioetanol (U.S. DOE, 2011) y biobutanol (Baral y Shah, 2016), respectivamente.

La Figura 2.4 esquematiza las diferentes subetapas del pretratamiento, dependientes del tipo de método utilizado y del tipo de residuo lignocelulósico. Como producto del pretratamiento suele obtenerse una mezcla de líquido y sólido, la hidrólisis se lleva a cabo sobre dicha mezcla o sobre la fracción sólida que se genera mediante separación, obteniéndose en este caso también una fracción líquida rica en pentosas. Mientras que en la producción de bioetanol el pretratamiento y la hidrólisis se centran en la producción de hidrolizados ricos en glucosa, en la producción de biobutanol toma también relevancia la recuperación de pentosas, ya que las bacterias del género *Clostridium* pueden consumir ambos tipos de monosacáridos (Amiri y Karimi, 2018).

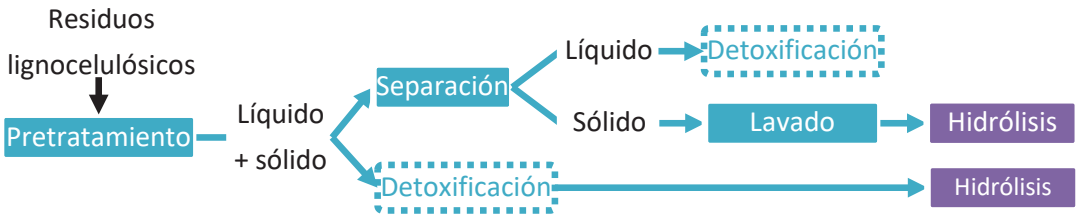

Figura 2.4. Etapas previas en la producción de bioetanol y biobutanol a partir de residuos lignocelulósicos. Adaptada de Amiri y Karimi (2018).

Durante el pretratamiento de los residuos lignocelulósicos pueden liberarse compuestos inhibidores de la posterior hidrólisis enzimática y fermentación (Amiri y Karimi, 2018). Estos compuestos pueden clasificarse en tres grupos: aldehídos derivados de azúcares, compuestos aromáticos y ácidos orgánicos y aldehídos de cadena corta, donde el tipo de inhibidor y la cantidad de estos dependen del material lignocelulósico pretratado y de las condiciones del método. Dos aldehídos

relevantes generados en la degradación de azúcares son el furfural (derivado de las pentosas) y el HMF (*hydroxymethylfurfural*, derivado de las hexosas). Ambas moléculas no afectan considerablemente a la hidrólisis enzimática, pero sí a los microorganismos, ya que inhiben enzimas como la alcohol deshidrogenasa, alteran el material genético, afectan a la formación de la membrana celular y generan especies reactivas del oxígeno. Por otra parte, los compuestos aromáticos, derivados principalmente de la lignina, se subdividen en compuestos fenólicos (vainillina, siringaldehido y coniferil aldehído), compuestos no fenólicos (ácido benzoico, cinamaldehído y alcohol bencílico) y benzoquinonas. Estos inhibidores afectan tanto a las celulasas como a la integridad de la membrana celular. Los compuestos fenólicos tienen un efecto tóxico superior al resto de inhibidores y pueden difundirse directamente a través de la membrana celular debido a su bajo peso molecular. Otro tipo de inhibidores son los ácidos orgánicos de cadena corta como el acético (derivado de los grupos acetilo de la hemicelulosa y la lignina), el fórmico (derivado del furfural y del HMF), el láctico (derivado de los azúcares) y el levulínico (derivado del HMF). Estas moléculas pueden difundirse a través de la membrana celular en su forma no disociada y disociarse dentro de la matriz celular, con un pH cercano a la neutralidad, incrementando la demanda energética como consecuencia del desacople del transporte de iones. También modifican la membrana celular y afectan a la síntesis de material genético. Los aldehídos de cadena corta como el formaldehido y el acetaldehído proceden de la degradación de la lignina e inhiben el crecimiento microbiano (Kim, 2018; Sjulander y Kikas, 2020).

La concentración de compuestos inhibidores presentes en la fracción sólida derivada del pretratamiento puede reducirse eficazmente mediante lavado, pero la fracción líquida o la mezcla de ambas fracciones en ocasiones requiere una etapa de detoxificación (Amiri y Karimi, 2018). Las técnicas de detoxificación son muy diversas y ninguna presenta un rendimiento eficaz sobre todos los compuestos. Algunas se centran en la eliminación de inhibidores mediante degradación química, adsorción en fase sólida, extracción líquido-líquido o nanofiltración. Otras se basan en el uso de hongos como *Coniochaeta ligniaria*, *Paecilpmyces variotii* o bacterias como *Urebacillus thermosphaericus* para eliminar biológicamente los inhibidores. Todos estos métodos pueden disminuir considerablemente la concentración de azúcares, afectando a la viabilidad global del proceso (Kim, 2018; Sjulander y Kikas, 2020). Una alternativa a estas técnicas consiste en aumentar la tolerancia microbiana a los inhibidores mediante la modificación de genes reguladores. También se han estudiado modificaciones genéticas que dan lugar a la producción o sobreproducción de enzimas que transforman los inhibidores en compuestos

químicos menos tóxicos (como oxidorreductasas y deshidrogenasas), de moléculas protectoras frente al estrés (como trehalosa, espermidina, tiorredoxina y glutatión) o de transportadores y bombas de eflujo. Otras modificaciones genéticas se centran en la membrana celular o en las rutas metabólicas. Dado que el genoma de la mayoría de los microorganismos productores de bioetanol y biobutanol no se ha estudiado en su totalidad, la ingeniería genética continúa presentando numerosas dificultades en este campo. Una estrategia que permite solventar este problema es la adaptación evolutiva, en la que se generan microorganismos tolerantes al sufrir mutaciones espontáneas en medios con inhibidores (Sjulander y Kikas, 2020).

Tras el pretratamiento, es necesario liberar los monosacáridos presentes en la fase sólida o en la mezcla de fases mediante una etapa de hidrólisis enzimática. Con tal finalidad se utilizan enzimas, tanto celulasas como hemicelulasas, que son producidas comercialmente a partir de hongos como *Trichoderma reseei*. Las distintas celulasas presentan, tal y como ilustra la Figura 2.5, acciones diferenciadas sobre los extremos reductores (celobiohidrolasas de tipo I) y no reductores (celobiohidrolasas de tipo II) de la celulosa, las regiones amorfas de la celulosa (endoglucanasas) y la disociación de celobiosa (β-glucosidasas) como disacárido de dos glucosas (Balan, 2014).

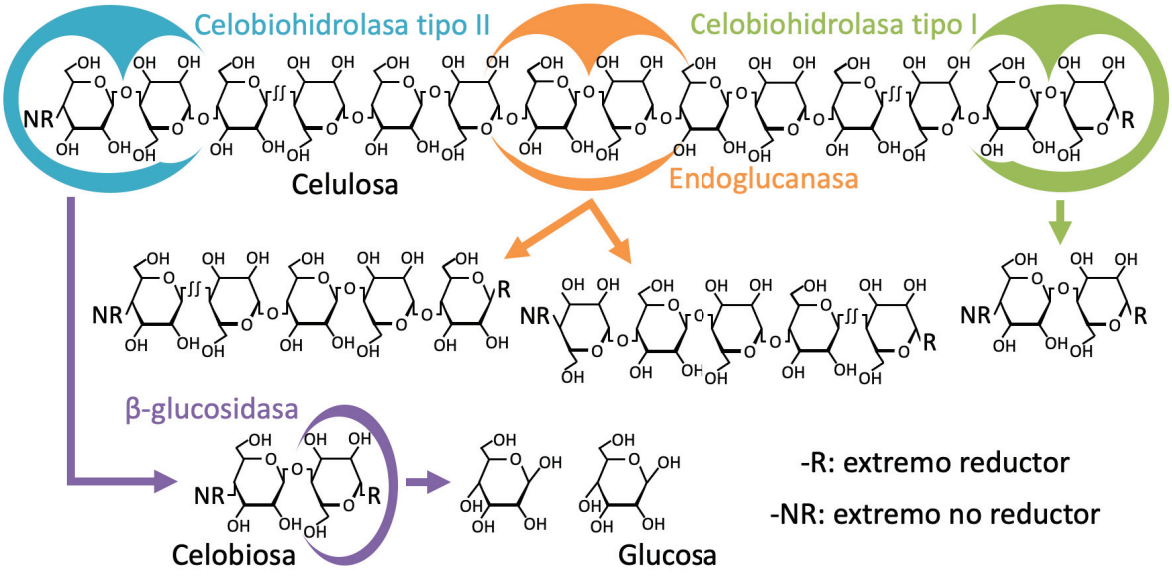

Figura 2.5. Enzimas implicadas en la hidrólisis de la celulosa. Adaptada de Bignell y col., (2011).

Ya que *T. reseei* no es un buen productor de glucosidasas, la hidrólisis enzimática se suele suplementar con β-glucosidasas obtenidas de *Aspergillus niger* (Domínguez-Escribà y Porcar, 2010). Una alternativa a la hidrólisis enzimática es la hidrólisis ácida, pero es menos sostenible con el medio ambiente, requiere más

energía, produce una mayor degradación de los azúcares y afecta a los microorganismos en la fermentación posterior (Martinez y col., 2001).

Otra de las desventajas que presenta la naturaleza recalcitrante de los residuos lignocelulósicos, junto con la necesidad de una etapa de pretratamiento, es el incremento de la cantidad de enzima requerida en la hidrólisis en comparación con los residuos ricos en almidón (Balan, 2014). El efecto del coste de las enzimas sobre la producción fermentativa a gran escala de biocarburantes a partir de residuos lignocelulósicos continúa siendo objeto de estudio, mientras que algunas evaluaciones técnico-económicas lo consideran como el mayor obstáculo para el desarrollo de esta tecnología, otras consideran que dicho efecto es menor. La diferencia encontrada en estas evaluaciones se debe a factores como el tipo de residuo lignocelulósico utilizado y el rendimiento de los biocarburantes (Klein-Marcuschamer y col., 2012). Pereira y col. (2018) determinaron que el coste de las enzimas supone entre el 20 y el 25% de los costes operacionales en la producción de bioetanol o biobutanol a partir de pulpa *Kraft* de eucalipto dependiendo del tipo de pretratamiento y del modo de operación. Las empresas que comercializan las enzimas han realizado un gran esfuerzo para reducir su coste y aumentar su actividad específica, no obstante, se requiere un mayor desarrollo basado en la identificación de nuevas enzimas con mayor actividad o con funciones diversas, en la producción de nuevas enzimas mediante ingeniería genética y en la evolución directa de las enzimas (Balan, 2014).

El contenido de los disolventes en el caldo de cultivo tras la etapa fermentativa es ≤10% (p/p), por lo que es necesario aplicar un proceso de recuperación y purificación para su uso como biocarburantes. El contenido final de bioetanol varía entre el 2.5 y el 10% (p/p), mientras que se requiere una pureza del 99.5% (p/p) para su uso (Sánchez y Montoya, 2013). El método que se ha utilizado tradicionalmente para llevar a cabo la deshidratación es la destilación, un proceso con alta demanda energética que precisa de concentraciones de bioetanol iniciales superiores a 4 – 5% (p/v) para ser viable económicamente (Balan, 2014; Chen y col., 2008). Este método consta de varias etapas, la primera se basa en la destilación para aumentar el contenido del disolvente al 50% (p/p). Posteriormente, en otra columna de destilación, se incrementa este porcentaje hasta el 90 – 92% (p/p). Para obtener bioetanol a una pureza del 99.5% (p/p) son necesarias etapas adicionales de destilación por cambio de presión, destilación azeotrópica, destilación extractiva, adsorción o pervaporación (Sánchez y Montoya, 2013). La concentración final de biobutanol en el caldo de cultivo suele ser menor al 1% (p/v) a consecuencia de la alta toxicidad de este disolvente (Ahlawat y col., 2019; Rochón y col., 2017). Una de las grandes limitaciones en la producción industrial de biobutanol reside en los

elevados requisitos energéticos de la recuperación de producto por destilación. Esto se debe, entre otras causas, a que el butanol posee una mayor temperatura de ebullición que el agua (118 °C), al contrario que el etanol (Veza y col., 2021). Se ha estimado que la recuperación de bioetanol en un proceso industrial que utiliza residuos lignocelulósicos como sustrato supone el 5% de los costes totales (U.S. DOE, 2011), en el caso del biobutanol este porcentaje puede llegar a alcanzar el 10% (Baral y Shah, 2016).

2.3. Residuos lignocelulósicos como sustrato en la producción fermentativa de biocarburantes

La biomasa lignocelulósica es el tipo de biomasa más abundante en la tierra, incluye los residuos producidos en actividades agrícolas, las plantas herbáceas (como el pasto de varilla y el césped), los árboles de madera dura (como álamo, eucalipto y roble) o madera blanda (como pino y abeto) y algunos residuos orgánicos municipales e industriales (Birgen y col., 2019; Isikgor y Becer, 2015). La valorización de residuos lignocelulósicos no se restringe únicamente a la potencial producción de biocarburantes, también pueden llegar a ser el punto de partida de más de 200 productos de valor añadido, ya sean sustancias químicas o polímeros sintéticos (Isikgor y Becer, 2015).

2.3.1. Principales residuos lignocelulósicos producidos en la agricultura

A modo de ejemplo, la Tabla 2.2 muestra la producción mundial en 2019 de los principales residuos agrícolas ricos en lignocelulosa junto con su composición en base a los tres principales componentes (celulosa, hemicelulosa y lignina). Tal y como se observa, la paja de arroz, material objeto de estudio en esta tesis doctoral, es uno de los residuos lignocelulósicos con mayor producción con 1133 millones de toneladas anuales. La Tabla 2.2 también presenta el porcentaje de azúcares potenciales de los residuos, es decir, la suma de los porcentajes de celulosa y hemicelulosa. En el caso de la paja de arroz dicha suma es del 64%, lo que demuestra el gran potencial de este material como fuente de azúcares fermentables.

Tabla 2.2. Producción mundial en 2019 (millones de toneladas), composición mayoritaria (%) y azúcares potenciales (%) de distintos residuos lignocelulósicos. Datos obtenidos de FAOSTAT (2019), Ibrahim y col. (2017), Kurian y col. (2013) y Putro y col. (2016).

Residuo	Producción (millones de toneladas)	Composición (%)			Azúcares potenciales (%)[f]
		Celulosa	Hemicelulosa	Lignina	
Rastrojo de maíz	1838[a]	31 – 40	20 – 25	11 – 25	51 – 65
Paja de trigo	1149[b]	35 – 39	22 – 30	12 – 16	57 – 69
Paja de arroz	1133[b]	48	16	7	64
Bagazo de caña de azúcar	546[c]	42	22	20	64
Mazorca de maíz sin grano	230[d]	45	33	15	78
Manojo vacío de aceite de palma	95[e]	39	21	19	60

[a] Cociente residuo/grano de 1.6. [d] Cociente residuo/grano de 0.2.
[b] Cociente residuo/grano de 1.5. [e] Cociente residuo/palma de 1.3.
[c] Cociente residuo/caña de 0.3. [f] Azúcares potenciales (%) = celulosa (%) + hemicelulosa (%).

2.3.2. Tipos de pretratamientos de residuos lignocelulósicos

Los distintos métodos usados en el pretratamiento de residuos lignocelulósicos pueden ser físicos (microondas), químicos (ácidos, líquidos iónicos, disolventes orgánicos, ozono y bases), fisicoquímicos (agua caliente presurizada y explosión con vapor o con dióxido de carbono) y biológicos (Balan, 2014; Putro y col., 2016). Independientemente del tipo de pretratamiento, un método adecuado debe de cumplir una serie de criterios como son: presentar una baja demanda energética, unos bajos costes de operación y la posibilidad de llevarse a cabo a escala industrial sin dificultades y minimizar la degradación de azúcares y la formación de compuestos inhibitorios (Kim, 2018).

A modo de resumen, en la Tabla 2.3 se muestran ejemplos de pretratamientos de distintos residuos lignocelulósicos junto con las condiciones del proceso y la fracción de interés, en todos los casos las fases sólida y líquida se separaron. La Tabla 2.3 también presenta la recuperación de sólidos y los rendimientos obtenidos tras la hidrólisis enzimática de la fracción sólida y, en la mayoría de los casos en los que se recuperó la fracción líquida del pretratamiento, se muestra la concentración detectada de inhibidores y monosacáridos presentes en la misma.

Tabla 2.3. Ejemplos de pretratamientos de diversos residuos lignocelulósicos.

Método	Sustrato	Condiciones del proceso	Carga de sólidos	Fracción de interés	Recuperación de sólidos
Microondas	Paja de arroz	320–680 W 20–30 min.	6–9% (p/v)	Sólida y líquida	-
Ácido diluido	Residuos de jardín	0.5–1.0% (p/p) H_2SO_4 140–160 °C 30 min.	10% (p/p)	Sólida y líquida	51–66%
Ácido diluido asistido por microondas	Posos de café gastado	0.5–1.5% (p/v) H_2SO_4 150–190 °C 5 min.	10% (p/v)	Sólida y líquida	54–90%
Líquido iónico	Paja de arroz	[Emim][OAc] 52–137 °C 18–372 min.	3.3–11.7% (p/p)	Sólida	63–95%
Ozono	Grano gastado de cervecera	2.7% (p/p) O_3 40–60% humedad 30 min.	20 g	Sólida	97%
Alcalino	Partes del tallo de maíz	0.5–2% NaOH 80–120 °C 60 min.	10% (p/v)	Sólida	50–70%
Alcalino	Grano gastado de cervecera	1–5% (p/p) NaOH 120 °C 30 min.	5–20% (p/p)	Sólida y líquida	39–61%
Alcalino	Grano gastado de cervecera	5% (p/p) H_2O_2 50 °C 60–180 min.	5–15% (p/p)	Sólida y líquida	43–67%
Agua caliente presurizada	Paja de arroz	0–1% (p/p) NaOH 140–180 °C 5–20 min.	10% (p/v)	Sólida y líquida	~40–57%
Biológico con *P. brevispora*	Rastrojo de maíz	1.7–7.3 mg inóculo/g 59–89% humedad 28 °C/42 días	36% (p/v)	Sólida	73–78%

Tabla 2.3. cont. Ejemplos de pretratamientos de diversos residuos lignocelulósicos.

Inhibidores en fracción líquida	Monosacáridos en fracción líquida	Rendimientos de hidrólisis		Referencia
		Frente sólido pretratado	Frente sólido original	
-	-	31–39% G[a] 15–20% X[a]	-	Ma y col. (2009)
3.7–6.2 g/L á. acético	1.5–6.5 g/L G 10.1–14.3 g/L X 2.2–3.4 g/L A y M	33–52% G	-	Farmanbordar y col. (2020)
0.1–0.2 g/L á. acético 0.1–3.0 g/L á. fórmico 0.1–1.9 g/L HMF 0.2–1.7 g/L fenoles	0.5–9.6 g/L G 5.3–25.2 g/L M	-	27–100% G	López-Linares y col. (2021)
-	-	33–100% G 25–83% X 4–57% A	-	Poy y col. (2021)
-	-	45–69% G 22–31% X y A	18–48% G 20–30% X y A	Fernández-Delgado y col. (2019)
-	-	-	35–87% G 35–70% X	Cai y col. (2016)
<1 mg/L furfural <1 mg/L HMF 1.2–3.4 g/L fenoles	1.0–12.1 g/L G 2.8–15.7 g/L X 0.6–8.3 g/L A	36–70% G 26–42% X y A	27–62% G 12–31% X y A	Fernández-Delgado y col. (2019)
<1 mg/L furfural <1 mg/L HMF 0.5–1.4 g/L fenoles	3.6–13.5 g/L G 3.5–13.0 g/L X 1.2–5.2 g/L A	72–99% G 24–52% X y A	55–63% G 14–28% X y A	Fernández-Delgado y col. (2019)
0.13–0.42 mg/L furfural 0.02–0.26 mg/L HMF	Hasta 5.9 g/L G Hasta 17.0 g/L X y A	-	Hasta 80% G	Imman y col. (2015)
-	-	24–31% G 10–12% X	-	Saha y col. (2017)

G: glucosa/X: xilosa/A: arabinosa/M: manosa.

[a] Se considera también la fracción líquida del pretratamiento.

Los métodos físicos de pretratamiento incluyen el uso de microondas, una técnica con tiempos de proceso cortos y requerimientos energéticos reducidos. En referencia a las desventajas, presenta un elevado coste del reactor y una escasa obtención de azúcares (Balan, 2014). Ma y col. (2009) lograron fraccionar el complejo lignina-hemicelulosa de la paja de arroz e incrementar el área superficial de la celulosa con unas condiciones de operación de microondas de 680 W, 24 minutos y 75 g/L de carga de sustrato. Dichos cambios incrementaron el rendimiento de la glucosa en base a la biomasa pretratada en un 30.6% tras la hidrólisis enzimática en comparación con la hidrólisis de la biomasa no pretratada. En el caso de la xilosa, el incremento del rendimiento fue del 43.3%. Por otro lado, la eliminación de lignina tras el pretratamiento fue moderada, de forma que el porcentaje de lignina soluble en ácido disminuyó únicamente de 2.1 a 1.9%.

Los pretratamientos químicos se basan en la acción de un determinado reactivo. Tras aplicar un ácido como clorhídrico o sulfúrico, la hemicelulosa se solubiliza en monosacáridos como la xilosa, mientras que la celulosa o bien se solubiliza directamente en glucosa o pierde cristalinidad y permanece disponible para ser hidrolizada. Uno de los grandes inconvenientes de esta técnica es la elevada producción de inhibidores (Balan, 2014). Farmanbordar y col. (2020) obtuvieron un líquido con 6.5 g/L de glucosa y 14.3 g/L de xilosa tras someter residuos de jardín a un pretratamiento con ácido sulfúrico diluido (1% p/p, 160 °C y 30 minutos), pero observaron en dicha fase altas concentraciones de ácido acético (6.2 g/L). López-Linares y col. (2021) optimizaron un pretratamiento en el que se combinó el uso de ácido sulfúrico diluido con microondas en posos de café gastado. La finalidad de su estudio era maximizar el rendimiento de glucosa en la hidrólisis de la fracción sólida y la liberación de monosacáridos procedentes de la hemicelulosa en la fracción líquida. Tras obtener las condiciones óptimas (161 °C y H_2SO_4 al 1.5% p/v), se logró recuperar el 100% de la glucosa del material original en la hidrólisis y se liberaron 2.0 g/L de glucosa y 24.7 g/L de manosa en el líquido del pretatamiento. A pesar de que la concentración de ácido acético en dicho líquido fue baja (0.2 g/L), se originaron otros inhibidores (0.2 g/L de ácido fórmico, 0.4 g/L de HMF y 0.3 g/L de compuestos fenólicos).

Los compuestos químicos como líquidos iónicos, disolventes orgánicos y ozono producen menos inhibidores en el pretratamiento que los ácidos, pero suelen presentar costes más elevados. Los líquidos iónicos, sales orgánicas con puntos de fusión menores a 100 °C, generan un eficaz fraccionamiento y disolución de los residuos lignocelulósicos. Su naturaleza no corrosiva y biodegradable y la posibilidad de recuperación completa son propiedades que los convierten en una alternativa ventajosa frente a otras sustancias químicas. Una de las principales desventajas de

estos compuestos es que pueden ser tóxicos para las enzimas y los microorganismos (Usmani y col., 2020). Investigaciones recientes realizadas en el seno de nuestro grupo de investigación (Poy y col., 2021) demostraron que el pretratamiento de paja de arroz con acetato de 1-etil-3-metilimidazolio en unas condiciones óptimas de 120 °C, 300 minutos y 5% (p/p) como carga de sólidos redujo el contenido en lignina a la mitad. Tras aplicar estas condiciones, en la hidrólisis enzimática el porcentaje de azúcares liberados del residuo pretratado fue del 98% frente al 19% obtenido a partir del residuo no pretratado. Otro compuesto químico neutro es el ozono, a pesar de que la ozonólosis es descrita como una técnica que permite la degradación selectiva de la lignina, Fernández-Delgado y col., 2019 no observaron cambios significativos en el grano gastado de cervecera tras someterlo a ozono (2.7% p/p) durante 30 minutos a temperatura ambiente y 40 – 60% de humedad.

El tratamiento con bases como hidróxido sódico, amoniaco o cal genera menos inhibidores y produce una mayor deslignificación que el tratamiento con ácidos, incrementando notablemente el área superficial de la celulosa. Junto con los ácidos, las bases eliminan la mayoría de grupos acetilo y ácidos urónicos de la hemicelulosa (Putro y col., 2016). Cai y col. (2016) obtuvieron porcentajes de deslignificación del 73 – 91% en distintas partes del tallo de maíz (flor, hoja, vástago, cáscara y mazorca) mediante un pretratamiento con hidróxido sódico al 2% (p/v) y 120 °C durante 60 minutos. A pesar de que estas condiciones favorecieron la posterior hidrólisis enzimática, también incrementaron la pérdida de celulosa y hemicelulosa, por lo que la recuperación de azúcares del material original fue menor que en condiciones más suaves. Fernández-Delgado y col. (2019) observaron que el pretratamiento alcalino de grano gastado de cervecera con hidróxido sódico o peróxido de hidrógeno puede originar una fracción líquida rica en azúcares (> 30 g/L). En este estudio, el mayor grado de deslignificación obtenido fue del 50% con hidróxido sódico (5% p/p, 120 °C, 30 minutos) y del 59% con peróxido de hidrógeno (5% p/p, 50 °C, 180 minutos), en ambos casos la carga de sólidos fue del 15% (p/p). En estas condiciones se recuperó en la hidrólisis el 55 – 62% de la glucosa y el 12 – 14 % de la xilosa y arabinosa del material original.

En los pretratamientos fisicoquímicos se trabaja en condiciones de alta temperatura y presión, por lo que se requiere un gran aporte energético. El método de agua caliente presurizada se basa en la disociación del agua en iones hidronio e hidróxido a alta temperatura (140 – 180 °C) y alta presión (20 bar), estos iones hidrolizan la hemicelulosa al romper los grupos acetilo y los ácidos urónicos (Imman y col., 2015). Esta técnica presenta las ventajas de generar pocos inhibidores, no requerir compuestos químicos y evitar la corrosión de los reactores (Qureshi y col., 2018). Imman y col. (2015) compararon el pretratamiento de paja de arroz con agua

caliente presurizada con o sin la adición de hidróxido sódico. Al añadir la base se incrementó la liberación de xilosa y arabinosa en el pretratamiento y se mejoró la posterior hidrólisis enzimática, resultando en un porcentaje de glucosa liberada en referencia al material original del 80% tras usar una temperatura de 140 °C y una concentración de hidróxido sódico del 0.25% durante 10 minutos.

Los pretratamientos biológicos son llevados a cabo con microorganismos capaces de degradar la lignina como los hongos de podredumbre blanca, parda o blanda y algunos tipos de bacterias como actinomicetos y proteobacterias. Los microorganismos con mayor potencial de biodeslignificación son los hongos de podredumbre blanca, capaces de producir peroxidasas y lacasas. Es un tipo de pretratamiento más lento y menos efectivo que el resto de las técnicas, sin embargo, no origina inhibidores y no está sujeto a condiciones de temperatura y presión extremas (Balan, 2014; Putro y col., 2016). Saha y col. (2017) optimizaron el pretratamiento de rastrojo de maíz con un hongo de podredumbre blanca, *Phlebia brevispora* NRRL-13108, productor de celulasas, xilanasas y enzimas degradadoras de lignina. Tras establecer las condiciones óptimas del proceso (84% de humedad y 2.5 mg de inóculo por gramo de biomasa), obtuvieron 442 gramos de azúcares por cada kilogramo de residuo pretratado tras la hidrólisis enzimática.

2.3.3. Paja de arroz como sustrato en la producción fermentativa de biocarburantes

El arroz es el cuarto cultivo con mayor producción a nivel mundial tras la caña de azúcar, el maíz y el trigo (FAOSTAT, 2019). Desde 1961 hasta 2019 la producción de arroz se ha incrementado en un 250%, por lo que actualmente se obtienen al año 755 millones de toneladas, principalmente en países asiáticos como China (28%), India (24%), Indonesia (7%) y Bangladesh (7%). En Europa se obtienen al año 4 millones de toneladas, siendo Italia (37%), Rusia (27%) y España (19%) los principales países productores. La paja de arroz es la fracción vegetativa que se obtiene en la cosecha del arroz, compuesta por láminas y vainas foliares, tallos y raquis de la panícula (Oladosu y col., 2016). Considerando un cociente paja/grano de 1.5 (Kurian y col., 2013), en 2019 se generaron 1133 millones de toneladas de paja de arroz en el mundo. Este residuo lignocelulósico puede utilizarse para el techado, cultivo de hongos, embalaje y para la fabricación de papel, cartón, alcohol y cuerdas. Sin embargo, debido a los costes adicionales de recolección y almacenamiento y al bajo potencial forrajero frente a la paja de trigo, los agricultores de los países asiáticos queman a campo abierto el 25 – 95% de la paja de arroz. La quema a campo abierto es una estrategia que permite reincorporar en la tierra parte de los nutrientes para

el siguiente ciclo de cultivo, pero supone una gran fuente de emisión de GHG (Gadde y col., 2009; Vivek y col., 2019).

Una de las características que convierten a la paja de arroz en un sustrato potencial para la producción fermentativa de biocarburantes es su bajo contenido en lignina (7%, Tabla 2.2) en comparación con otros residuos lignocelulósicos (11 – 25%, Tabla 2.2). Sin embargo, presenta un alto contenido en cenizas y sílice (8 – 20%). La acumulación de sílice proporciona resistencia mecánica a la planta y le protege de patógenos y metales pesados. En la hidrólisis enzimática, esta molécula se une de forma no específica a las enzimas, disminuyendo el rendimiento relativo a la liberación de azúcares. El bajo contenido en lignina y el alto contenido en sílice determinan el tipo de pretratamiento adecuado para la paja de arroz. El pretratamiento alcalino es particularmente eficaz sobre los materiales con escasa lignina frente a otros métodos de pretratamiento (Satlewal y col., 2018). Entre los compuestos químicos utilizados en el pretratamiento alcalino de la paja de arroz, el hidróxido sódico conduce a una deslignificación más eficiente (Hosseini y col., 2012), permitiendo eliminar también la sílice de este residuo lignocelulósico al formar ambos compuestos un complejo (Mukherjee y col., 2018). De esta forma, la eliminación de lignina y sílice, la condición no corrosiva de las bases, la escasa degradación de azúcares y la reducida producción de compuestos inhibidores hacen del pretratamiento alcalino con hidróxido sódico uno de los métodos más adecuados para usar sobre la paja de arroz (Satlewal y col., 2018; Vivek y col., 2019). Por otra parte, se ha demostrado que el uso de microondas sin (Ma y col., 2009) o con (Singh y col., 2014) hidróxido sódico como catalizador logra romper y eliminar la matriz de sílice de este residuo. Ma y col. (2009) observaron la eliminación del 7.5% de la sílice junto con el enriquecimiento en celulosa y hemicelulosa como los principales cambios en la composición del material tras su pretratamiento con unas condiciones de operación de 680 W, 24 minutos y 75 g/L de carga de sustrato. Junto con la eliminación de sílice, el pretratamiento con microondas presenta una serie de ventajas que lo convierten en otra posible alternativa para usar sobre paja de arroz: tiempos de proceso reducidos, eliminación eficaz de grupos acetilo de la hemicelulosa y escasa producción de compuestos inhibidores (López-Linares y col., 2019). Con la finalidad de aumentar la viabilidad económica del proceso, y considerando que las condiciones de operación del pretratamiento afectan a la susceptibilidad del sustrato a la hidrólisis y posterior fermentación (Bajpai, 2016b), es necesario optimizar dichas condiciones.

Uno de los principales factores a considerar en el aprovechamiento de la paja de arroz para la producción de bioenergía y bioproductos es su disponibilidad local. Esto se debe a los altos costes de transporte derivados de la naturaleza voluminosa

de este residuo lignocelulósico (Domínguez-Escribà y Porcar, 2010). En este sentido, el Parque Natural de la Albufera, donde se genera en torno al 14% de la producción nacional de arroz y donde se producen 75000 – 90000 toneladas de paja de arroz al año (Ribó y col., 2017), se encuentra a tan solo 10 km del área metropolitana de Valencia (Viana y col., 2008). Esta es una de las principales causas por las que la paja de arroz se seleccionó como la materia prima a utilizar en el desarrollo de esta tesis doctoral. El Parque Natural de la Albufera es un icónico humedal costero de la Península Ibérica y uno de los más importantes de la costa mediterránea occidental, situado específicamente en la llanura litoral que forman las desembocaduras de los ríos Júcar y Turia. Fue declarado Parque Natural en 1986 y alberga una gran diversidad biológica debido a los distintos sistemas ambientales que lo forman (Benedito y col., 2011). Cuenta también con un rico legado cultural como consecuencia de la actividad pesquera, agrícola y cinegética ligada a la presencia humana que se remonta a tiempos prehistóricos (Ferrando y Jambrino, 2014). La gestión tradicional de la paja de arroz en el parque era la quema a campo abierto durante los meses de septiembre y octubre. Un estudio realizado en Valencia concluyó que esta actividad emite, junto con los GHG, diversos contaminantes atmosféricos como partículas, metales e hidrocarburos aromáticos policíclicos que suponen un grave riesgo para la salud de la población (Viana y col., 2008). Actualmente, esta práctica está prohibida en muchos países del mundo y la Política Agraria Común de la Unión Europea ha incrementado gradualmente la exigencia de otros modos de gestión. Una práctica alternativa es el triturado e incorporación al suelo, ni esta actividad ni el aprovechamiento del residuo en biorrefinerías suponen una disminución de la calidad del suelo y del rendimiento en la producción de grano en comparación con la quema a campo abierto (Ribó y col., 2017). Sin embargo, la incorporación del residuo al suelo conduce a la generación de grandes cantidades de metano y de ácido sulfhídrico, a enfermedades de los cultivos y a un aumento de la mortalidad de la fauna relacionado con la putrefacción (Domínguez-Escribà y Porcar, 2010; Ribó y col., 2017). En referencia a la producción de bioenergía y bioproductos, actualmente el grupo Naturgy está estudiando la viabilidad de producir biogás utilizando paja de arroz del Parque Natural de la Albufera en una planta piloto de biometanización (Naturgy, 2019). Alternativamente a la producción de biogás, la producción de biobutanol a partir de paja de arroz puede ser una estrategia prometedora en la transformación del sistema energético y en la reducción potencial del impacto ambiental derivado del cultivo del arroz.

2.4. Bibliografía

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3. PRODUCCIÓN DE BIOBUTANOL A PARTIR DE RESIDUOS LIGNOCELULÓSICOS MEDIANTE FERMENTACIÓN ABE

3.1. Fermentación ABE

La fermentación ABE es una fermentación anaerobia llevada a cabo por bacterias del género *Clostridium*, donde la proporción másica típica de disolventes acetona:butanol:etanol es 3:6:1. Estas bacterias son grampositivas, anaerobias estrictas mayoritariamente, tienen forma de varilla y forman esporas (Jones y Woods, 1986). En las últimas tres décadas las especies *Clostridium* más utilizadas en estudios de fermentación ABE en modo discontinuo han sido *C. beijerinckii*, *C. acetobutylicum*, *C. saccharobutylicum* y *C. saccharoperbutylacetonicum* (Birgen y col., 2019). La mayor parte de la información relativa a estas bacterias se basa en el estudio de la cepa *C. acetobutylicum* ATCC 824, por lo que muchas ideas generales no son aplicables a todas las cepas y especies (Patakova y col., 2013).

La Figura 3.1 esquematiza la cronología de los hitos históricos de la fermentación ABE. La producción de butanol mediante fermentación fue descubierta en 1862 por Louis Pasteur a partir de un cultivo mixto ("Vibrion butyrique") del que probablemente formaban parte las especies bacterianas *C. butyricum* y *C. acetobutylicum*. El primer cultivo puro productor de butanol lo aisló Albert Fitz en 1876, quien denominó a este cultivo como *Bacillus butylicus*, llevando a cabo una serie de estudios que fueron el punto de partida de numerosas investigaciones realizadas a finales del siglo XIX y a lo largo del siglo XX. Probablemente *B. butylicus* era *C. acetobutylicum*, cuyo nombre fue validado en 1926. La explotación industrial de la fermentación ABE comenzó durante la Primera Guerra Mundial debido a la necesidad de aumentar la producción de acetona para obtener cordita, compuesto propulsor de cartuchos y proyectiles. Para ello fue necesaria la patente de Jaim Weizmann de 1915 relativa a la fermentación de biomasa rica en almidón a partir de un cultivo mixto del que formaba parte *C. acetobutylicum*. Desde 1920 a 1982 muchos países produjeron butanol a escala industrial y, hasta 1950, dos de las terceras partes de la producción se correspondían a la fermentación ABE. Sin embargo, el abaratamiento del petróleo tras la Segunda Guerra Mundial y el incremento del coste de las melazas, sustrato que se utilizaba comúnmente en esta industria, causaron el cese gradual de la producción de biobutanol (Dürre, 2008, 2007). En los últimos años las crisis cíclicas del petróleo y la necesidad mundial de sustituir a los combustibles fósiles por alternativas renovables han incrementado el interés en la fermentación ABE. En 2017 la empresa Green Biologics comenzó a producir industrialmente biobutanol en Minnesota (Estados Unidos), pero la planta se cerró en 2019 debido a la falta de fondos (IEA, 2020). Actualmente, la empresa Gevo (Estados Unidos) produce

isobutanol de primera generación a partir de maíz, utilizando una levadura modificada genéticamente (Gevo, 2019).

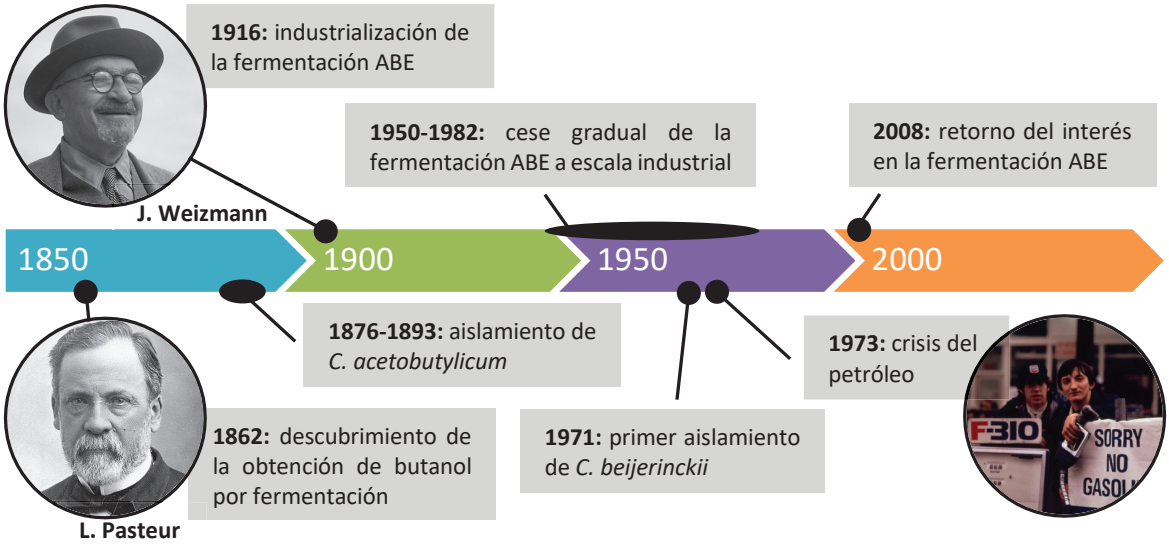

Figura 3.1. Cronología de los hitos históricos de la fermentación ABE. Elaboración propia a partir de datos obtenidos de Dürre (2007) y Veza y col. (2021).

La fermentación ABE presenta dos fases: acidogénica y solventogénica. En la fase acidogénica, la bacteria crece exponencialmente y los monosacáridos (hexosas y pentosas) son transformados en ácidos (acético y butírico), hidrógeno y dióxido de carbono, disminuyendo el pH del caldo de fermentación. Posteriormente, en la fase solventogénica, el crecimiento cesa y comienza la esporulación mientras que los monosacáridos se siguen consumiendo y los ácidos, a su vez, son reasimilados en disolventes. Esta última fase está inducida por una serie de factores como el descenso del pH externo a valores comprendidos entre 4.5 y 5 (dependiendo de la cepa y las condiciones de cultivo) y el aumento de la concentración de ácidos no disociados. Además, el cambio de fase requiere el mantenimiento de un correcto gradiente de pH a través de la membrana bacteriana, la disposición de fuentes de carbono y nitrógeno en concentraciones no limitantes, la presencia de factores limitantes del crecimiento bacteriano como sulfato, fosfato y magnesio en concentraciones adecuadas y una correcta temperatura (Jones y Woods, 1986). Para *C. acetobutylicum*, el factor que más influye en el cambio de fase es la concentración de ácido butírico no disociado en etapas tempranas de la fermentación, donde concentraciones comprendidas entre 0.2 y 1.5 g/L inducen el inicio de la solventogénesis (Yang y col., 2013). Para *C. beijerinckii* se ha observado este cambio a partir de concentraciones de ácidos no disociados (acético y butírico) comprendidas entre 4 y 9 mM (Maddox y col., 2000).

La Figura 3.2 muestra la ruta metabólica que componen las dos fases en las que se divide la fermentación ABE, mientras que la acidogénesis está sombreada en azul, la solventogénesis lo está en verde. En la ruta metabólica se destacan los genes que codifican las principales enzimas.

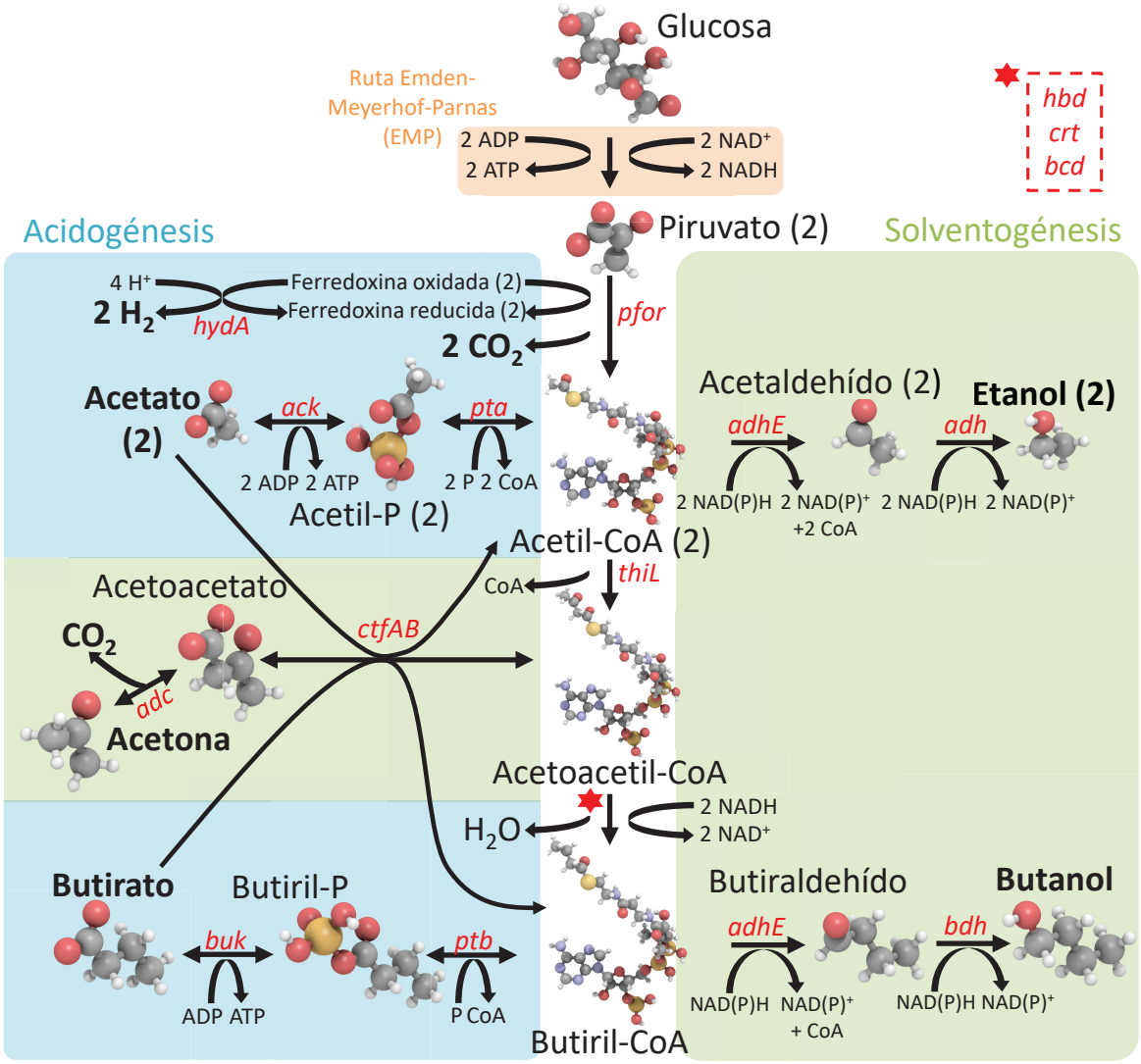

Figura 3.2. Vía metabólica de la fermentación ABE y principales genes involucrados: *pfor*, piruvato:ferredoxin óxidorreductasa; *hydA*, hidrogenasa; *pta*, fosfotransacetilasa; *ack*, acetato quinasa; *thiL*, tiolasa; *hbd*, β-hidroxibutiril-CoA deshidrogenasa; *crt*, crotonasa; *bcd*, butiril-CoA deshidrogenasa; *ptb*, fosfotransbutirilasa; *buk*, butirato quinasa; *adhE*, aldehído deshidrogenasa; *adh*, alcohol deshidrogenasa; *ctfAB*, acetoacetil-CoA:acetato/butirato:CoA transferasa; *adc*, acetoacetato descarboxilasa; *bdh*, butanol deshidrogenasa. Adaptada de Abo y col. (2019), Kushwaha y col. (2019) y Moon y col. (2016). Las moléculas se construyeron usando Avogadro V.1.2.0 (http://avogadro.cc/).

Aparte de esta ruta metabólica general, se ha observado que otras cepas bacterianas generan productos diferentes, por ejemplo, algunas cepas de *C. beijerinckii* poseen el gen *sadh*, el cual codifica una alcohol deshidrogenasa secundaria dependiente de NADPH que permite reducir la acetona en isopropanol (Abo y col., 2019). Las especies *C. tetanomorphum*, *C. carboxidivorans* y *C. thermosaccharolyticum* no producen acetona, dando lugar a una mezcla de butanol y etanol (Patakova y col., 2013).

Una de las principales ventajas de la fermentación ABE como bioproceso es que las bacterias del género *Clostridium* pueden metabolizar diversas fuentes de carbono. Pueden consumir totalmente algunos monosacáridos (glucosa, manosa y fructosa), disacáridos (sacarosa y lactosa), dextrinas y almidón y parcialmente otros monosacáridos (xilosa, arabinosa, galactosa), alcoholes (glicerol y manitol), rafinosa e insulina (Kushwaha y col., 2019). No obstante, las vías por las que las bacterias del género *Clostridium* transportan y consumen estos compuestos continúan siendo objeto de estudio. Se ha demostrado que las cepas *C. acetobutylicum* ATCC 824 y *C. beijerinckii* NRRL B-598 transportan hexosas y disacáridos al citoplasma mediante el sistema fosfotransferasa dependiente del fosfoenolpiruvato (*Phosphotransferase System*, PTS). Este sistema está compuesto por enzimas localizadas en la membrana celular (permeasa IIC/IID) y en el citoplasma (enzima I responsable de la transferencia de grupos fosforilo, PtsI; proteína con histidina, PtsH; enzima IIA y enzima IIB). Otras vías de transporte independientes del sistema PTS presentes en estas cepas son los transportadores ABC (*ATP-Binding Cassette*) y simportadores de protones, responsables también del transporte de pentosas (Patakova y col., 2019, 2013; Vasylkivska y col., 2019). Tras incorporarse en el citoplasma, las hexosas se metabolizan mediante la ruta Embden-Meyerhoff-Parnass (EMP), también llamada glucólisis. Las pentosas son metabolizadas en la ruta de las pentosas fosfato (*Pentose Phosphate Pathway*, PPP) antes de entrar en la glucólisis (Vasylkivska y col., 2019), lo que implica una serie de reacciones adicionales que disminuyen la velocidad de consumo en comparación con la de las hexosas (Finneran y Popovic, 2018). Recientemente se ha descubierto una nueva ruta metabólica de pentosas denominada ruta de la fosfocetolasa (*Phosphoketolase Pathway*, PKP) que es independiente de la glucólisis (Amiri y Karimi, 2018). Al contrario que *C. acetobutylicum*, *C. beijerinckii* tiene localizados la mayoría de los genes relativos al metabolismo de xilosa en un gran clúster y posee un mayor número de estos genes (Birgen y col., 2019). El gran potencial que presenta *C. beijerinckii* en referencia al consumo de xilosa es una de las principales causas por la que se seleccionó como la especie bacteriana a utilizar en el desarrollo de esta tesis doctoral.

Las bacterias del género *Clostridium* metabolizan preferentemente las hexosas cuando crecen en mezclas de hexosas y pentosas debido a diferencias en el transporte y en las rutas de metabolización y al mecanismo denominado represión catabólica por carbono (*Carbon Catabolite Repression*, CCR). En referencia a la represión catabólica por carbono, en muchos casos se ha observado que la xilosa no comienza a consumirse hasta que la concentración de glucosa en el medio no disminuye por debajo de un valor límite (Birgen y col., 2019; Finneran y Popovic, 2018). Este mecanismo limita el uso de azúcares y, por ende, la producción de disolventes ABE, pudiendo dar lugar a un crecimiento diaúxico de la población bacteriana y/o a una fase de latencia extensa, incrementando así el tiempo del proceso (Abo y col., 2019; Birgen y col., 2019). Está regulado por la proteína CcpA, codificada por el gen *ccpA*, que interacciona con la proteína PtsH del sistema PTS y actúa como reguladora transcripcional. Además, la enzima II del sistema PTS, codificada por el gen *glcG*, también interviene en la represión. La inactivación de ambos genes mediante técnicas de ingeniería metabólica puede mejorar el consumo de xilosa (Amiri y Karimi, 2018; Wen y col., 2019b). Otras formas de lograr la utilización simultánea de hexosas y pentosas se centran en aplicar diversas estrategias de alimentación, semihidrólisis, crecimiento previo, cultivos mixtos y suplementación con micronutrientes (Abo y col., 2019; Birgen y col., 2019). En estudios de fermentaciones de mezclas de monosacáridos, las cepas *C. beijerinckii* BA101 y *C. acetobutylicum* ATCC 824 consumieron simultáneamente la glucosa y diversas pentosas (manosa, arabinosa y xilosa), pero la glucosa se consumió más rápidamente y/o a un mayor porcentaje que las pentosas (Ezeji y col., 2007; Raganati y col., 2015; Shao y Chen, 2015).

Un fenómeno no deseado en la fermentación ABE es la esporulación de las bacterias *Clostridium*, debido a que supone un consumo adicional de energía y carbono no destinado a la producción de disolventes y a que las esporas maduras no presentan un metabolismo activo. La solventogénesis y la esporulación poseen reguladores comunes en algunas cepas *Clostridium*. Un regulador transcripcional común es la proteína Spo0A que, tras activarse probablemente por fosforilación mediada por histidina quinasas, activa y reprime una serie de genes dando lugar a una reacción en cascada. Sin embargo, la correlación entre solventogénesis y esporulación continúa siendo objeto de discusión en el campo de la fermentación ABE y existen cepas como *C. beijerinckii* NRRL B-598 y *C. acetobutylicum* DSM 1791 y ATCC 824 capaces de producir disolventes sin esporular (Kolek y col., 2017).

La fase solventogénica finaliza cuando la concentración total de disolventes alcanza valores tóxicos para la bacteria (~2 % p/v), siendo pues la inhibición por producto una de las grandes limitaciones para llevar a cabo la fermentación ABE a

escala industrial. El disolvente que presenta una mayor toxicidad para la bacteria es el butanol, cuya concentración límite se ha fijado en 1% (p/v), aproximadamente (Ahlawat y col., 2019; Rochón y col., 2017). La toxicidad del butanol radica en su naturaleza hidrofóbica, de modo que incrementa la fluidez de la membrana celular al perturbar los fosfolípidos presentes en esta y promueve la autolisis celular al desencadenar la producción de autolisinas. Al incrementarse la fluidez de la membrana se ve afectada la actividad ATPasa asociada a la membrana, el gradiente de pH, el transporte de hierro y aminoácidos y el sistema PTS. La célula responde modificando los lípidos de la membrana, donde se incrementa la proporción de ácidos grasos de cadena larga, y expresando genes de proteínas de choque térmico (Ezeji y col., 2010; Jones y Woods, 1986). Además de la inhibición por producto, en la fermentación ABE puede producirse inhibición por sustrato a partir de concentraciones de azúcares en el medio (>100 g/L) que afectan al crecimiento bacteriano (Ibrahim y col., 2018).

Un suceso ocasional que se ha observado en la fermentación ABE en modo discontinuo sin control de pH es el *acid crash*, caracterizado por una producción de ácidos en exceso mientras que la producción de disolventes finaliza tempranamente tras el cambio de fase. La causa de este suceso es la presencia de altas concentraciones de ácidos (acético y butírico) en su forma no disociada al combinarse una alta velocidad de producción de ácidos en etapas tempranas de la fermentación y un bajo pH. Para C*. beijerinckii*, la concentración límite de ácidos no disociados varía entre 57 y 60 mM (Maddox y col., 2000).

3.2. Fermentación ABE a partir de residuos lignocelulósicos

El uso de residuos lignocelulósicos en la fermentación ABE resuelve uno de los principales problemas que dificultan la producción de biobutanol a nivel industrial, el alto coste del sustrato. A diferencia de las fermentaciones ABE que utilizan residuos lignocelulósicos, el precio del sustrato en fermentaciones a partir de biomasa alimentaria rica en almidón puede suponer más del 70% del coste total (Cui y col., 2021).

La sacarificación y fermentación separada (*Separate Hydrolysis and Fermentation*, SHF) en modo discontinuo es la configuración más ampliamente estudiada en la fermentación ABE a partir de residuos lignocelulósicos por ser la más sencilla a nivel operativo. Se trata de un proceso en el que la fermentación tiene lugar tras la hidrólisis enzimática en otro reactor independiente. En la Tabla 3.1 se detallan ejemplos de procesos SHF en modo discontinuo a partir de diversos residuos lignocelulósicos en los que se han utilizado distintos métodos de

pretratamiento, distintas cargas de sólidos y de enzima en la hidrólisis y distintas cepas *Clostridium* en la fermentación. Mientras que en la mayoría de ejemplos se fermentó el hidrolizado obtenido a partir de la fase sólida derivada del pretratamiento (Amiri y col., 2014; Borah y col., 2019; Chi y col., 2019; Fernández-Delgado y col., 2019; Qi y col., 2019; Sun y col., 2020; Xu y col., 2020), en algunos se fermentó también el líquido derivado del pretratamiento (López-Linares y col., 2021, 2019) y en otros no se dio separación de fases entre el pretratamiento y la hidrólisis enzimática (López-Linares y col., 2020; Wang y col., 2020). En función de todos estos factores se obtuvo un amplio rango de concentraciones (2.3 – 13.1 g/L), rendimientos (0.15 – 0.27 g/g de azúcar consumido) y productividades (0.011 – 0.218 g/L h) de biobutanol. Otro parámetro que aparece en la Tabla 3.1 es el cociente entre el butanol producido y la biomasa original utilizada, el cual varía entre 45 y 113 g/kg. Esta variable cuantifica el aprovechamiento del residuo original, teniendo en cuenta el balance de masas de las tres etapas secuenciales: pretratamiento, hidrólisis y fermentación.

La producción de biobutanol a partir de residuos lignocelulósicos continúa presentando una serie de desafíos que limitan la producción, el rendimiento y la productividad de este disolvente. Entre todos los factores que limitan este proceso se pueden destacar la inhibición por producto, la formación de múltiples productos (otros disolventes, ácidos y gases), la utilización incompleta de pentosas por parte de las bacterias, la obtención de una baja densidad célular, la inhibición por sustrato y la necesidad de múltiples etapas de proceso (Birgen y col., 2019; Ibrahim y col., 2017). Además, la baja concentración de butanol en el caldo de fermentación (∼1% p/v) encarece considerablemente su recuperación y purificación, siendo esta una de las principales causas del elevado coste de obtención de butanol por fermentación ABE (Abo y col., 2019). Frente a estos desafíos se están investigando numerosas estrategias como son: la mejora de las cepas *Clostridium*, el uso de cocultivos, la optimización del medio, la optimización de etapas previas, el uso del modo de fermentación semicontinuo y continuo, el uso de configuraciones de alta densidad celular y la integración de etapas del proceso. En la Figura 3.3 se relacionan los principales desafíos de la producción de biobutanol a partir de residuos lignocelulósicos con las estrategias que están siendo investigadas para poder solucionarlos. Tal y como se puede observar, una misma estrategia permite afrontar distintos retos.

Tabla 3.1. Ejemplos de procesos SHF a partir de residuos lignocelulósicos.

Sustrato	Pretratamiento	Fracción de interés	Carga de sólidos pretratados	Carga de enzima	Microorganismo
Paja de arroz	Organosolv con etanol	Sólida	8% (p/p)	25 FPU/g sólido	*C. acetobutylicum* NRRL B-591
Malezas invasoras	Ácido sulfúrico diluido y alcalino asistido por ultrasonidos	Sólida	4.2% (p/v)	135 FPU/g sólido	*C. acetobutylicum* MTCC 11274
Paja de arroz	Alcalino con hidróxido sódico	Sólida[e]	12% (p/v)	50 FPU/g	*C. beijerinckii* NCIMB 8052
Grano gastado de cervecera	Alcalino con peróxido de hidrógeno	Sólida	10% (p/p)	15 FPU/g sólido	*C. beijerinckii* DSM 6422
Grano gastado de cervecera	Hidrotérmico asistido por microondas	Sólida / Líquida[b]	5% (p/v)	15 FPU/g sólido	*C. beijerinckii* DSM 6422
Paja de trigo	Sulfito de amonio diluido	Sólida	10.5% (p/v)	10 FPU/g sólido	*C. acetobutylicum* ATCC 824
Grano gastado de cervecera	Ácido sulfúrico diluido asistido por microondas	Sólida y líquida[a]	4.8% (p/v) / 7.8% (p/v)	15 FPU/g sólido	*C. beijerinckii* DSM 6422
Pasto de varilla	Hidrotérmico	Sólida[c]	14%	50 FPU/g glucano	*C. beijerinckii* NCIMB 8052
Rastrojo de maíz	Ácido sulfúrico diluido	Sólida y líquida[d]	12.5% (p/v)	15 FPU/g sólido	*C. acetobutylicum* CICC 8016
Mazorca de maíz	Disolvente eutéctico profundo basado en etilamina	Sólida	7.7% (p/v)	50 FPU/g celulasa	*C. saccharo-butylicum* DSM 13864
Posos de café gastado	Ácido sulfúrico diluido asistido por microondas	Sólida / Líquida	15% (p/v) / -	15 FPU/g sólido / -	*C. beijerinckii* DSM 6422

[a] Tras la hidrólisis enzimática, el hidrolizado se detoxificó con carbono activo.

[b] El líquido de pretratamiento se detoxificó con carbono activo.

[c] Se utilizó biocarbón de cedro rojo como fuente de nutrientes, compuesto tampón y detoxificante.

[d] Tras la hidrólisis enzimática, el hidrolizado se detoxificó con columna de intercambio aniónico.

[e] La hidrólisis enzimática se llevó a cabo en modo semicontinuo.

Tabla 3.1. cont. Ejemplos de procesos SHF a partir de residuos lignocelulósicos.

Producción de butanol/ ABE (g/L)	Rendimiento de butanol/ ABE (g/g azúcar)	Productividad de butanol/ ABE (g/L h)	Conversión de biomasa en butanol/ ABE (g/kg)	Referencia
7.1/10.5	-	0.099/0.146	70/103	Amiri y col. (2014)
5.8/9.6	0.15/0.38[2]	0.048/0.080	102/168	Borah y col. (2019)
6.2/9.0	0.24/0.35[1]	0.172/0.250[3]	96/142	Chi y col. (2019)
10.9/13.7	0.27/0.34[1]	0.114/0.143	45/56	Fernández-Delgado y col. (2019)
8.3/11.3	0.26/0.35[1]	0.086/0.118[3]	46/62	López-Linares y col. (2019)
2.3/2.7	0.17/0.20[1]	0.011/0.032[3]		
11.3/17.8	-	0.105/0.164	84/133	Qi y col. (2019)
8.2/11.8	0.26/0.37[1]	0.198/0.285[3]	113/162	López-Linares y col. (2020)
10.8/16.0	0.21/0.32[1]	0.175/0.259[3]	91/138	
13.1/19.2	0.22/0.32[1]	0.218/0.320	-	Sun y col. (2020)
6.9/10.3	0.24/0.36[1]	0.096/0.143	-	Wang y col. (2020)
10.4/15.8	0.19/0.30[2]	0.150/0.220	137/208[4]	Xu y col. (2020)
6.7/10.4	0.21/0.33[1]	0.140/0.216	95/151	López-Linares y col. (2021)
3.6/5.8	0.20/0.33[1]	0.074/0.121		

[1] Rendimiento basado en la concentración de azúcares consumidos.

[2] Rendimiento basado en la concentración de azúcares presentes en el medio de cultivo.

[3] Productividad calculada con el tiempo al que se obtiene la máxima concentración de producto.

[4] Conversión basada en biomasa pretratada.

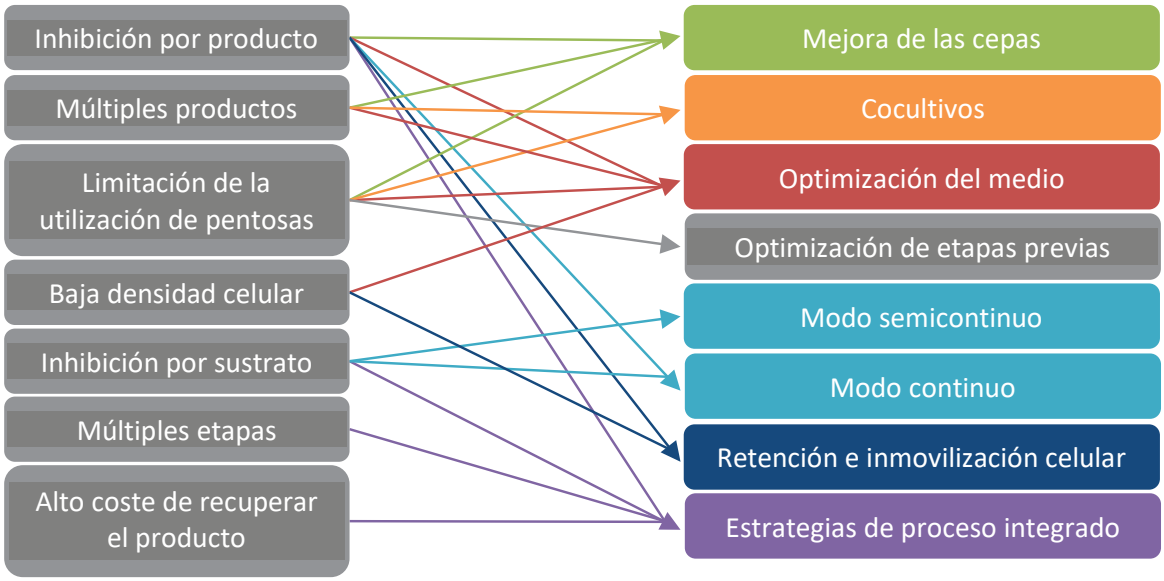

Figura 3.3. Retos de la producción de biobutanol a partir de residuos lignocelulósicos y estrategias para afrontarlos. Elaboración propia a partir de datos obtenidos de Ibrahim y col. (2017), Maiti y col. (2016), Finneran y Popovic (2018) y Abo y col. (2019).

La mejora de las cepas *Clostridium* puede llevarse a cabo mediante técnicas de mutagénesis aleatoria como la mutagénesis química y la evolución adaptativa en laboratorio y de forma directa a partir de ingeniería metabólica basada en recombinación homóloga, uso de ácido ribonucleico antisentido y uso de intrones del grupo II (Birgen y col., 2019; Xue y col., 2017). Numerosos estudios han utilizado la ingeniería metabólica para sobreexpresar genes involucrados en la ruta metabólica del butanol, bloquear genes involucrados en la producción de otros productos como acetona, sobreexpresar o bloquear genes relacionados con la resistencia a la toxicidad del butanol y sobreexpresar o bloquear genes relacionados con la captación y el metabolismo de los azúcares para favorecer el consumo de xilosa (Ibrahim y col., 2017). Sin embargo, se han obtenido mejores resultados con la mutagénesis química y la evolución adaptativa que con la ingeniería metabólica debido al limitado desarrollo de las herramientas de manipulación genética y al insuficiente estudio de estas bacterias en el campo de la biología molecular (Xue y col., 2017). Desafortunadamente, e independientemente del tipo de técnica utilizada, las cepas mutadas pierden sus características fenotípicas en el transcurso de varios ciclos de cultivo (Ibrahim y col., 2018). En estos últimos años se está realizando un gran esfuerzo en el sector de la biotecnología para introducir correctamente la ruta de producción de butanol en otros microorganismos como *E. coli* y *S. cerevisiae* y para aplicar la novedosa tecnología CRISPR (*Clustered Regularly Interspaced Short Palindromic Repeats*)-Cas, la cual permite la edición a gran escala del genoma de las bacterias (Xue y col., 2017).

En cualquier fermentación llevada a cabo a nivel industrial, el uso de cultivos puros suele ser la opción más viable, debido a que las condiciones de proceso son más simples y los productos finales se obtienen con mayor pureza y no son consumidos por otros microorganismos (Finneran y Popovic, 2018). Una alternativa al uso de cultivos puros es el uso de cocultivos de diferentes microorganismos que interaccionan entre sí y realizan funciones ecológicas complementarias. Las ventajas de usar cocultivos formados por dos especies *Clostridium* distintas o por bacterias *Clostridium* y otros microorganismos en la fermentación ABE son la ampliación del espectro de sustratos metabolizables, la protección de las bacterias *Clostridium* frente al oxígeno o compuestos inhibidores, la mejora de la producción de butanol y la formación de nuevos productos (Cui y col., 2021; Du y col., 2020).

En referencia a la optimización del medio, en numerosos estudios se ha observado un efecto positivo sobre la producción, rendimiento y/o proporción de butanol sobre otros productos tras suplementar el medio de la fermentación ABE con fuentes de carbono (glucosa y glicerol), fuentes de nitrógeno (extracto de levadura y peptona), ácidos (acético, butírico y láctico), portadores de electrones (metil viológeno, rojo neutro, azul de metileno, dimetilsulfóxido y antraquinona-2,6-disulfonato), cofactores sumideros de electrones (citrato férrico), sustancias tampón (hidróxido de calcio, carbonato de calcio y potasio hidrogenofosfato) y minerales (sulfato de magnesio, sulfato de hierro y sulfato de zinc). Estos compuestos, entre otros efectos, incrementan el consumo de xilosa, la tolerancia al butanol y/o la tolerancia a inhibidores procedentes de los residuos lignocelulósicos (Finneran y Popovic, 2018; Maiti y col., 2016). Cabe destacar que la composición óptima del medio es dependiente de la cepa bacteriana y que en los ensayos de optimización no suele tenerse en cuenta el efecto de los compuestos presentes en los hidrolizados lignocelulósicos, ya que suelen realizarse a partir de medios sintéticos. Por otra parte, también se suelen optimizar distintas variables de la fermentación ABE como el pH, factor que afecta a la promoción y mantenimiento de la fase solventogénica, al consumo de xilosa, a la densidad celular y a la selectividad del butanol (Finneran y Popovic, 2018; Patakova y col., 2018).

Otra estrategia que permite afrontar algunos de los retos de este proceso es la optimización de etapas previas (pretratamiento e hidrólisis). El uso de una correcta configuración referente a las condiciones de operación de ambas etapas conduce a una serie de beneficios que no se limitan a la reducción de costes de proceso, sino que también incluyen la mejora del desarrollo de la fermentación ABE. Por ejemplo, la optimización de factores de la hidrólisis como la carga enzimática y el tipo de enzimas utilizadas posibilita evitar el mecanismo CCR (Zhao y col., 2018).

3.3. Configuraciones avanzadas de proceso en la fermentación ABE a partir de residuos lignocelulósicos

En las últimas décadas se está trabajando en el desarrollo de configuraciones avanzadas que permitan mejorar o reemplazar el proceso SHF en modo discontinuo en la producción de biobutanol a partir de residuos lignocelulósicos (Figura 3.3). En esta sección se resumen los principales avances en relación con: modos de fermentación, sistemas de alta densidad celular e integración de procesos.

3.3.1. Modos de fermentación

La fermentación ABE puede llevarse a cabo en modo discontinuo, semicontinuo y continuo. El modo de operación determina la producción, la productividad total y la recuperación de producto. El modo discontinuo, en el que se fermenta todo el sustrato en un solo lote, es una configuración simple, fácil de controlar y que reduce el riesgo de contaminación y de degeneración celular (Abo y col., 2019; Ibrahim y col., 2018). La degeneración celular es un suceso en el que una cepa concreta pierde la capacidad de entrar en fase solventogénica al sufrir un cambio genético tras un largo periodo de tiempo (Maddox y col., 2000). No obstante, esta configuración se caracteriza por presentar bajas producciones de disolventes ABE a causa de las limitaciones en la carga de sustrato (Ibrahim y col., 2018) y bajas productividades a causa de los tiempos muertos de preparación y de latencia (Vees y col., 2020). El modo discontinuo es ampliamente utilizado en la fermentación ABE a pequeña escala en los estudios de laboratorio y a gran escala en la industria europea (Vees y col., 2020). Alternativamente al modo discontinuo, la alimentación de sustrato en modo semicontinuo y continuo está ganando importancia por su capacidad de incrementar la cantidad de biomasa utilizada.

El modo semicontinuo, en el que se añade secuencialmente el sustrato mientras que este es consumido simultáneamente por las bacterias, permite utilizar grandes cantidades de biomasa sin causar inhibición por azúcares o CCR. Esta configuración debe de combinarse con la recuperación integrada de producto, ya sea en serie o *in situ*, para solventar la inhibición por producto asociada a la fermentación de concentraciones elevadas de sustrato (Birgen y col., 2019; Ibrahim y col., 2018; Kushwaha y col., 2019; Vees y col., 2020). Mientras que la recuperación en serie se basa en un circuito separado a partir del cual se recuperan las células bacterianas y un efluente empobrecido en disolventes ABE (Figura 3.4 a), el caldo de cultivo no sale del biorreactor en la recuperación *in situ* (Figura 3.4 b). La combinación del modo semicontinuo con la recuperación integrada de producto

aumenta la producción con respecto a la fermentación ABE en modo discontinuo (Ibrahim y col., 2018; Veza y col., 2021). Sin embargo, los tiempos muertos del modo semicontinuo son similares a los del modo discontinuo, por lo que la productividad total del proceso es semejante (Vees y col., 2020).

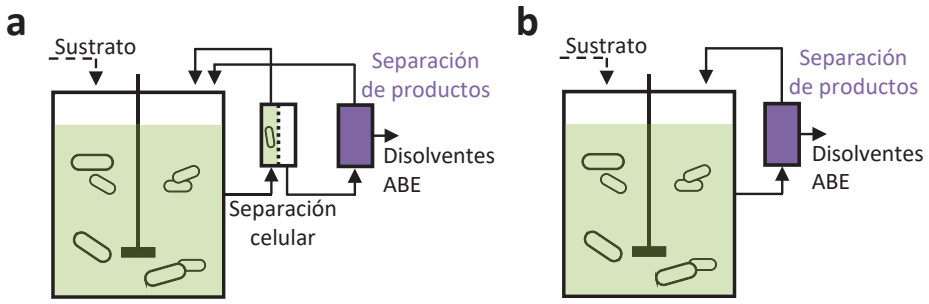

Figura 3.4. Recuperación integrada de producto en serie (a) e *in situ* (b) en la fermentación ABE en modo discontinuo. Adaptada de Vees y col. (2020).

El modo continuo, en el que se añade el sustrato y se retira el caldo de la fermentación con los disolventes ABE de manera ininterrumpida, reduce la inhibición por producto, permite mantener una alta estabilidad operacional y puede presentar altas productividades (Birgen y col., 2019; Ibrahim y col., 2018; Kushwaha y col., 2019; Vees y col., 2020; Veza y col., 2021). Como desventajas da lugar a bajas producciones de disolventes ABE y requiere un especial control para evitar la degeneración de la cepa, la contaminación microbiana y la entrada de oxígeno (Vees y col., 2020; Veza y col., 2021). La naturaleza de la fermentación ABE, compuesta por dos fases, y la limitación de la velocidad de dilución, debido a las bajas velocidades de crecimiento celular, son los principales problemas del modo continuo con células en suspensión (Ibrahim y col., 2018; Vees y col., 2020).

3.3.2. Sistemas de alta densidad celular

Las estrategias de retención e inmovilización celular permiten incrementar la densidad celular en el modo continuo al desacoplar el crecimiento bacteriano y la velocidad de dilución. Una mayor densidad celular en la fermentación ABE suele traducirse en una mayor producción (Vees y col., 2020). Por otra parte, la inmovilización celular en el modo discontinuo permite llevar a cabo ciclos de repetición que pueden incrementar la productividad (Kushwaha y col., 2019).

La retención celular se lleva a cabo en un retentostato, una modificación del quimiostato o reactor continuo de tanque agitado (*Continuous Stirred Tank Reactor*, CSTR) en la que las células bacterianas se retienen mediante filtración (Figura 3.5 a). En esta estrategia, la velocidad de crecimiento se controla a partir de la purga del caldo de cultivo y se puede lograr un consumo completo del sustrato. Sin embargo,

es una técnica compleja, con riesgos elevados de contaminación, en la que las células sufren estrés mecánico y en la que se retienen células inactivas y partículas del sustrato, especialmente si se utilizan hidrolizados lignocelulósicos que suelen contener material particulado inerte (Vees y col., 2020).

Los sistemas de inmovilización celular protegen a las células de fuerzas mecánicas, compuestos inhibitorios y degeneración genética. La inmovilización puede llevarse a cabo por atrapamiento en membrana, encapsulación en matriz polimérica y unión a material sólido por enlaces covalentes, adsorción y/o enlaces iónicos. Los CSTR con partículas libres (Figura 3.5 b) y los reactores de lecho fijo (*Packed Bed Reactor*, PBR, Figura 3.5 c) son los sistemas de inmovilización comúnmente utilizados (Vees y col., 2020).

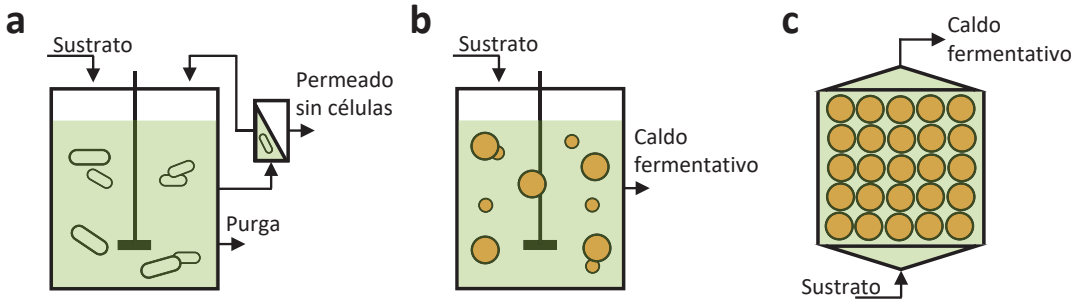

Figura 3.5. CSTR con retención celular (a) y con partículas libres de células inmovilizadas (b) y PBR (c). Adaptada de Vees y col. (2020).

3.3.3. Integración de procesos

El uso de residuos lignocelulósicos en la fermentación ABE implica una serie de etapas previas como el pretratamiento y la hidrólisis enzimática y una etapa posterior de recuperación de producto. Con la finalidad de aumentar la viabilidad económica y la productividad total de la producción de biobutanol a nivel industrial se han desarrollado diferentes configuraciones de proceso integrado que combinan estas etapas:

-Recuperación *in situ* de producto (*Separate Hydrolysis, Fermentation and Recovery*, SHFR).

-Sacarificación y fermentación simultánea (*Simultaneous Saccharification and Fermentation*, SSF).

-Sacarificación y fermentación simultánea con recuperación *in situ* de producto (*Simultaneous Saccharification, Fermentation and Recovery*, SSFR).

-Bioproceso consolidado (*Consolidated Bioprocessing*, CBP).

La Figura 3.6 esquematiza las distintas combinaciones que caracterizan cada una de las configuraciones nombradas.

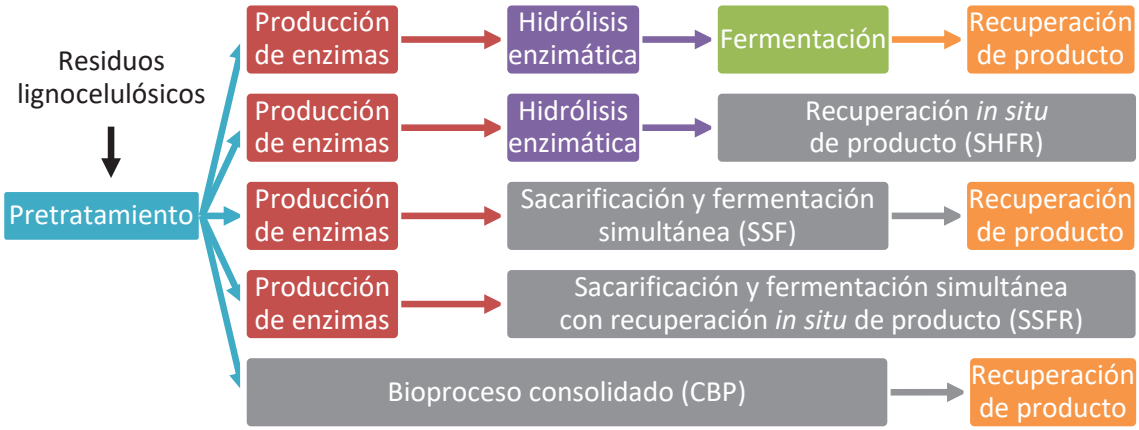

Figura 3.6. Estrategias de integración de etapas de fermentación ABE a partir de residuos lignocelulósicos. Adaptada de Ibrahim y col. (2017).

El proceso SHFR surge de la combinación de la configuración SHF y de la recuperación *in situ* de producto. La recuperación *in situ* de producto es especialmente necesaria en el modo semicontinuo para evitar la inhibición por producto, pero también se puede aplicar al modo continuo. Entre las operaciones unitarias de separación que se emplean se encuentran el arrastre por gas, la extracción líquido-líquido, la adsorción, la pervaporación y la perstracción. El arrastre por gas es un método simple y barato en el que el caldo de cultivo es burbujeado con los gases de la fermentación (hidrógeno y dióxido de carbono). Los productos arrastrados son posteriormente recuperados mediante condensación (Figura 3.7 a). La extracción líquido-líquido es un método con gran selectividad que se basa en la utilización de un disolvente muy miscible con el producto (Figura 3.7 b). Como desventajas es una técnica compleja, con altos costes y que requiere el uso de disolventes no tóxicos para las bacterias como el alcohol oleico. La adsorción es un método que se divide en dos etapas. En la primera, el producto es adsorbido por materiales como el carbono activo, zeolitas y resinas de intercambio iónico. Posteriormente, el adsorbente se regenera con vapor de agua dando lugar a una disolución concentrada de producto. Como desventajas requiere una etapa previa de micro o ultrafiltración para evitar el ensuciamiento, puede presentar baja selectividad y altos costes y, en el caso del modo continuo, requiere dos o más columnas. La pervaporación y la perstracción son métodos que se basan en el uso de membranas. En la pervaporación se emplea una membrana hidrofóbica por la que los productos pasan de una fase líquida (caldo de fermentación) a una fase gaseosa (permeado) en base a una diferencia de presión (Figura 3.7 c). Las

membranas habitualmente empleadas son de PDMS (*polydimethylsiloxane*) o de POMS (*polyoctylmethylsiloxane*). La utilización de membranas aumenta el coste del proceso y el ensuciamiento supone un problema al no ser posible la limpieza. La perstracción es una ampliación de la extracción líquido-líquido en la que las células bacterianas son separadas del caldo de fermentación como etapa previa a la aplicación del disolvente, por lo que pueden usarse disolventes tóxicos para las células (Vees y col., 2020).

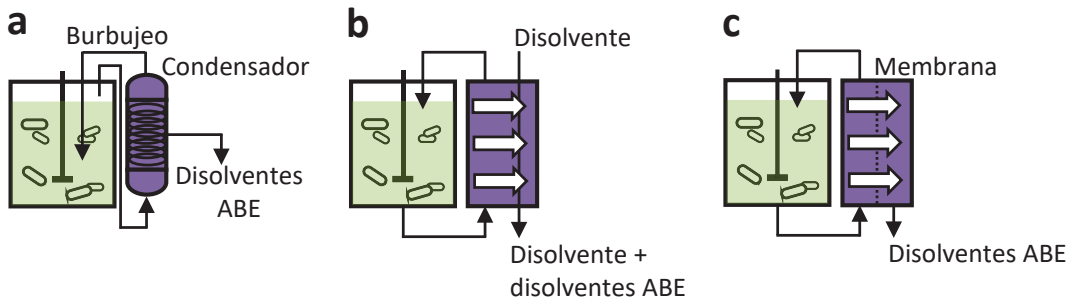

Figura 3.7. Operaciones unitarias de recuperación *in situ* de producto: a, arrastre por gas; b, extracción líquido-líquido; c, pervaporación. Adaptada de Lee y col. (2008).

En el proceso SSF, la hidrólisis enzimática y la fermentación ABE se llevan a cabo simultáneamente en el mismo recipiente. Esta estrategia reduce la inhibición de la β-glucosidasa y del crecimiento bacteriano por parte de la glucosa, ya que los azúcares son consumidos a medida que se liberan en el caldo de fermentación. La atenuación o eliminación de la inhibición por glucosa incrementa la conversión de residuos lignocelulósicos en disolventes ABE. Uno de los principales problemas del proceso SSF es la diferencia entre los valores óptimos de temperatura (45 – 60 °C) y pH (4.5 – 5.5) para las enzimas y los valores óptimos de temperatura (30 – 37 °C) y pH (4.5 – 6.0) para las especies *Clostridium*, lo cual aumenta la carga de enzimas requerida en el proceso. Otro problema es que, al contrario que en el proceso SHF en el que los azúcares pueden concentrarse antes de la fermentación, la concentración de azúcares en el proceso SSF está condicionada por la carga de sustrato (Ibrahim y col., 2018, 2017).

El proceso SSFR surge de la combinación de la configuración SSF y de la recuperación *in situ* de producto. Este proceso permite evitar tanto la inhibición por sustrato como la inhibición por producto. La eliminación de la inhibición por producto da lugar a una mayor producción de disolventes ABE en comparación con el proceso SSF (Ibrahim y col., 2017). Se trata de una estrategia muy atractiva desde el punto de vista económico (Qureshi y col., 2020). La combinación de la configuración SSFR con el modo de operación semicontinuo es una prometedora estrategia que puede permitir el uso de grandes cargas de residuo lignocelulósico

en la producción de biobutanol. Hasta la fecha, no se han desarrollado procesos SSF o SSFR en modo semicontinuo para la obtención de biobutanol, la causa principal es que la adición estéril de sustrato en el reactor continúa siendo un reto técnico a solventar (Ibrahim y col., 2018). No obstante, algunos estudios han demostrado la viabilidad de la alimentación secuencial de paja de arroz (Shengdong y col., 2006) y rastrojo de maíz (Wang y col., 2013) en procesos SSF en modo semicontinuo para la producción de bioetanol por *S. cerevisiae*.

En el proceso CBP, las etapas de producción de enzimas, hidrólisis y fermentación ABE se llevan a cabo simultáneamente en el mismo recipiente. A tal efecto se utilizan microorganismos con la capacidad de producir las enzimas y fermentar los azúcares. Esta estrategia reduce la inhibición por sustrato e implica el ahorro correspondiente a las enzimas hidrolíticas. Al contrario que en las fermentaciones ABE a partir de biomasa rica en almidón, en el caso de los residuos lignocelulósicos no se puede utilizar una única bacteria sino un cocultivo. Esto se debe a que las bacterias del género *Clostridium* que producen disolventes ABE o no expresan celulasas o lo hacen en un bajo grado (Ibrahim y col., 2018). Dos de las especies celulolíticas que pueden usarse junto con las especies solventogénicas son *C. thermocellum* y *C. cellulovorans* (Kiyoshi y col., 2015; Tomita y col., 2019; Wen y col., 2014). Por otra parte, numerosos estudios han modificado genéticamente la cepa *C. cellulovorans* ATCC 743B con la finalidad de que produzca butanol a partir de la expresión de genes heterólogos procedentes de *C. acetobutylicum* (Bao y col., 2019; Ou y col., 2017; Wen y col., 2019a; Yang y col., 2015).

3.4. Ejemplos de configuraciones avanzadas de proceso en la fermentación ABE a partir de residuos lignocelulósicos

En la Tabla 3.2 se presentan ejemplos relativos al desarrollo y aplicación de las mencionadas configuraciones avanzadas a diversos residuos lignocelulósicos.

Tabla 3.2. Ejemplos de configuraciones avanzadas aplicadas en fermentación ABE a partir de residuos lignocelulósicos.

Configuración	Sustrato	Pretratamiento	Carga de sólidos pretratados	Microorganismo
SHFR en semicontinuo inmovilizado	Bagazo de sorgo dulce	Alcalino con hidróxido sódico	25% (p/v)[a]	*C. acetobutylicum* ABE 1201
SHFR en continuo con células libres	Paja de trigo	Ácido sulfúrico diluido	20% (p/p)	Cepa GBL-B patentada por "Green Biologics" (Abingdon, UK)
Continuo con células libres	Salvado de arroz	Ácido sulfúrico diluido	12% (p/v)[b]	*C. acetobutylicum* YM1
Continuo inmovilizado	Virutas de madera de abeto	Sulfito ácido y organosolv con etanol	14% (p/v)[c]	*C. acetobutylicum* DSM 792
SSF	Rastrojo de maíz	Alcalino con hidróxido sódico	7.0% (p/v)	*C. saccharobutylicum* DSM 13864
SSF	Paja de trigo	Sulfito de amonio diluido	9.0% (p/v)	*C. acetobutylicum* ATCC 824
SSF	Rastrojo de maíz	Ácido sulfúrico diluido y alcalino	4.8% (p/v)[d]	*C. acetobutylicum* L7(GlcG)
SSFR	Rastrojo de maíz	Ácido sulfúrico diluido	8.6% (p/v)[e]	*C. beijerinckii* P260
CBP	Paja de arroz	Alcalino con hidróxido sódico	4.0% (p/v)	*C. saccharo-perbutylacetonicum* N1-4[f]
CBP	Mazorca de maíz sin grano	Alcalino con hidróxido sódico	7.0% (p/v)	*C. beijerinckii* NCIMB 8052[g]

[a] La hidrólisis enzimática se llevó a cabo en modo semicontinuo y el hidrolizado se concentró.
[b] La mezcla tras el pretratamiento se detoxificó con carbono activo.
[c] La mezcla tras el pretratamiento se trató con distintas etapas de acondicionamiento y se detoxificó con columna de intercambio aniónico (XAD-4).
[d] La fermentación se suplementó con líquido de pretratamiento detoxificado con hidróxido de calcio.
[e] La mezcla tras el pretratamiento se detoxificó con hidróxido de calcio.
[f] Cocultivo con *C. thermocellum* NBRC 103400.
[g] Cocultivo con *C. cellulovorans* ATCC 743B.

Tabla 3.2. cont. Ejemplos de configuraciones avanzadas aplicadas en fermentación ABE a partir de residuos lignocelulósicos.

Producción de butanol/ ABE (g/L)	Rendimiento de butanol/ ABE (g/g azúcar consumido)	Productividad de butanol/ ABE (g/L h)	Conversión de biomasa en butanol/ ABE (g/kg)	Referencia
27.0/41.4	0.20/0.32	0.150/0.230	-	Wen y col. (2018)
12.9/18.3	0.16/0.23	0.180/0.250[1]	-	Van Hecke y De Wever (2017)
6.9/12.4	0.24/0.43	0.136/0.248[2]	-	Al-Shorgani y col. (2019)
~4.50/7.59	~0.16/0.27	~2.900/4.860[3]	-	Survase y col. (2011)
12.3/19.2	-	0.257/0.400	175/273[4]	Dong y col. (2016)
12.6/19.8	-	0.088/0.138	110/173	Qi y col. (2019)
10.8/18.2	-	0.225/0.379	-	Wu y col. (2021)
11.6/20.8	0.22/0.39	0.193/0.340	-	Qureshi y col. (2014)
5.5/-	-	0.033/-	-	Kiyoshi y col. (2015)
8.3/11.8	-	0.104/0.148	-	Wen y col. (2014)

[1] Velocidad de dilución de 0.0137 h^{-1}.
[2] Velocidad de dilución de 0.02 h^{-1}.
[3] Velocidad de dilución de 0.64 h^{-1}.
[4] Conversión basada en biomasa pretratada.

En referencia al proceso SHFR, la Tabla 3.2 incluye un ejemplo de esta estrategia en modo semicontinuo con inmovilización mediante tallos de bagazo (Wen y col., 2018) y otro ejemplo referente al modo continuo con células libres (Van Hecke y De Wever, 2017). Wen y col. (2018) lograron la asimilación de 153.7 g/L de azúcares por parte de *C. acetobutylicum* ABE 1201 tras alimentar secuencialmente el sistema con hidrolizado de bagazo de sorgo dulce pretratado con hidróxido sódico y utilizar arrastre por gas para recuperar el producto. De esta forma, obtuvieron una producción acumulativa de butanol de 27 g/L tras 180 horas de fermentación, resultando en una productividad de 0.150 g/L h. Van Hecke y De Wever (2017) obtuvieron una menor producción de butanol (12.9 g/L) y una mayor productividad (0.180 g/L h) tras llevar a cabo el proceso SHFR en modo continuo durante 127 horas a partir de hidrolizado de paja de trigo pretratada con ácido sulfúrico y recuperación de producto mediante pervaporación con membranas de POMS. El modo continuo con células libres (Al-Shorgani y col., 2019) y con células inmovilizadas en pulpa de madera (Survase y col., 2011) también ha sido estudiado utilizando residuos lignocelulósicos sobre los que no se realizó una etapa de hidrólisis tras el pretratamiento. Al-Shorgani y col. (2019) compararon la fermentación ABE por *C. acetobutylicum* YM1 en modo discontinuo y en modo continuo a partir de salvado de arroz pretratado con ácido sulfúrico diluido. La aplicación del modo continuo, cuya duración fue de 300 horas, mejoró la productividad de butanol en un 18% frente al modo discontinuo, pero la producción se redujo en un 17%. En comparación con los procesos SHFR anteriormente nombrados, la productividad de butanol obtenida en este estudio fue algo menor (0.136 g/L h) debido a la baja producción (6.9 g/L). Una de las posibles causas de la reducida producción de butanol es la baja concentración de azúcares en la mezcla obtenida tras el pretratamiento (40.2 g/L), mientras que en los otros casos el sustrato presentaba una concentración de azúcares >100 g/L. A pesar de que Survase y col. (2011) prescindieron de la hidrólisis enzimática para fermentar virutas de madera de abeto, tras pretratar este residuo lignocelulósico fue necesario un complejo proceso de acondicionamiento (evaporación, arrastre por vapor, encalado y oxidación catalítica) y detoxificación (columna de intercambio aniónico XAD-4) para hacerlo fermentable. En la fermentación, llevada a cabo en un reactor de columna operado durante aproximadamente 20 días, se obtuvo una elevada productividad de butanol (∼2.9 g/L h) por parte de *C. acetobutylicum* DSM 792 como consecuencia de la alta densidad celular tras aplicar una estrategia de inmovilización. No obstante, la obtención de estos resultados requirió, junto con las etapas de acondicionamiento y detoxificación, la dilución del líquido de pretratamiento y la suplementación con 25 g/L de glucosa para asegurar un correcto crecimiento de *C. acetobutylicum* DSM

792, lo que demuestra la dificultad que presenta este tipo de material para su aprovechamiento en la fermentación ABE.

En referencia a la configuración SSF, en la mayoría de los experimentos presentados se realizó una etapa de prehidrólisis enzimática (2 – 12 horas) con la finalidad de disminuir la viscosidad del sistema y favorecer la transferencia de materia (Dong y col., 2016; Wu y col., 2021). Esta etapa previa también permitió liberar una cantidad inicial de azúcares para asegurar un correcto crecimiento bacteriano. La comparación entre el proceso SHF y el proceso SSF en estos estudios concluyó que la principal ventaja de la configuración integrada es la mejora de la productividad total de butanol en un 14 – 189%, calculada al considerar también la duración de la etapa de hidrólisis en el proceso SHF (48 – 72 horas) y la duración de la etapa de prehidrólisis en el proceso SSF (Dong y col., 2016; Qi y col., 2019; Wu y col., 2021). Wu y col. (2021) utilizaron la cepa *C. acetobutylicum* L7(GlcG), la cual presenta una modificación genética que le permite mantener altas velocidades de consumo de azúcar, de crecimiento y de producción de disolventes ABE a elevadas temperaturas (39 – 45 °C). De esta forma, el proceso SSF se llevó a cabo a 42 °C, favoreciendo la actividad enzimática sobre rastrojo de maíz pretratado con ácido sulfúrico diluido y amoniaco. En cuanto a la combinación del proceso SSF con la recuperación *in situ* de producto, Qureshi y col. (2014) informaron que la configuración SSFR mediante la aplicación de vacío permitió, al contrario que el proceso SSF, el consumo completo de los azúcares liberados de rastrojo de maíz pretratado con ácido sulfúrico diluido. De esta forma, se obtuvo un incremento del 29 y del 57%, respectivamente, en la producción y productividad de butanol por *C. beijerinckii* P260. En comparación con las configuraciones anteriormente nombradas, las productividades de butanol obtenidas en los procesos SSF y SSFR presentan mayoritariamente valores elevados (0.193 – 0.256 g/L h) incluso sin considerar el tiempo empleado en las etapas previas a la fermentación. Hasta la fecha, las configuraciones SSF y SSFR no han sido aplicadas en la producción de biobutanol a partir de paja de arroz como sustrato.

En la Tabla 3.2 se incluyen dos ejemplos del proceso CBP (Kiyoshi y col., 2015; Wen y col., 2014). El cocultivo de *C. thermocellum* NBRC 103400 y *C. saccharoperbutylacetonicum* N1-4 generó 5.5 g/L de butanol a partir de paja de arroz pretratada con hidróxido sódico tras 7 días de fermentación (Kiyoshi y col., 2015), resultando en una productividad de butanol muy reducida (0.033 g/L h). La suplementación con celulasa comercial (100 U/g sólido) incrementó la producción de butanol a 6.9 g/L. Wen y col. (2014) obtuvieron una productividad mayor (0.104 g/L h) a partir de mazorca de maíz sin grano pretratada con hidróxido sódico tras optimizar las condiciones del cocultivo de *C. cellulovorans* ATCC 743B y *C. beijerinckii*

NCIMB 8052. Las condiciones óptimas (inoculación simultánea de ambas cepas, cociente *C. beijerinckii*:*C. cellulovorans* en el inóculo de 1:10 v/v, control del pH en 7 durante las primeras 24 horas) dieron lugar a la producción de 8.3 g/L de butanol en 80 horas.

Los resultados previos referentes a la producción de biobutanol a partir de residuos lignocelulósicos mediante el proceso SSF y la ausencia de estudios relativos a la aplicación de esta configuración integrada a paja de arroz suscitaron el desarrollo de esta tesis doctoral.

3.5. Bibliografía

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4. OBJETIVOS

El objetivo general de la presente tesis doctoral es el estudio del proceso de sacarificación y fermentación simultánea (SSF) como una configuración alternativa al proceso convencional de sacarificación y fermentación separada (SHF) en la producción de biobutanol a partir de paja de arroz, utilizando *Clostridium beijerinckii* DSM 6422 como cepa bacteriana. Este objetivo general se ha concretado en los siguientes objetivos parciales:

1. Estudio comparativo de los procesos SSF y SHF a partir de paja de arroz pretratada mediante hidrotermólisis asistida por microondas en base a la eficiencia de la producción de biobutanol.
2. Evaluación del pretratamiento alcalino con hidróxido sódico, como alternativa a la hidrotermólisis asistida por microondas, con la finalidad de incrementar la accesibilidad de la celulosa y la hemicelulosa de la paja de arroz a las enzimas hidrolíticas.
3. Desarrollo de una configuración avanzada de SSF que integra la recuperación *in situ* de producto (SSFR) y que opera en modo de alimentación semicontinuo con el fin de incrementar la productividad de biobutanol, empleando las condiciones de pretratamiento alcalino previamente optimadas.

Los objetivos parciales se desglosan en los objetivos específicos descritos a continuación:

El estudio comparativo de los procesos SSF y SHF incluye:

- Análisis de los efectos sobre la producción de biobutanol de los parámetros: pH inicial, concentración de extracto de levadura y concentración de hierro en el medio de fermentación de los procesos SSF y SHF, tipo de tampón en la hidrólisis del proceso SHF y carga enzimática en el proceso SSF.
- Optimización del proceso SSF en base a los resultados derivados del estudio preliminar anterior para maximizar la producción de biobutanol, empleando como sustrato paja de arroz pretratada mediante hidrotermólisis asistida por microondas.

La evaluación del pretratamiento alcalino de la paja de arroz comprende:

- Análisis del efecto de la temperatura y tiempo de reacción, de la concentración de hidróxido sódico y de la carga de sólidos en el pretratamiento sobre la conversión de biomasa en biobutanol en el proceso SHF.
- Optimización del pretratamiento alcalino para maximizar la conversión de biomasa en biobutanol en base a los resultados derivados del estudio preliminar anterior.

El desarrollo del proceso SSFR en modo de alimentación semicontinuo contiene:

- Optimización del proceso SSF a partir de paja de arroz sometida a pretratamiento alcalino para maximizar la productividad de biobutanol.
- Evaluación del proceso SSFR en modo semicontinuo como configuración no aplicada hasta la fecha en la producción de biobutanol, utilizando el arrastre por gas como operación unitaria en la separación *in situ* de producto.
- Mejora de la viabilidad económica del proceso SSFR en modo semicontinuo, estudiando el efecto que tiene una reducción de la carga enzimática y de los componentes del medio sobre la producción de biobutanol.

5. MATERIALES, MÉTODOS Y PLAN DE TRABAJO

El presente capítulo incluye la descripción de los distintos materiales, equipos, técnicas experimentales y herramientas estadísticas utilizadas en los tres capítulos que constituyen la parte central de esta tesis doctoral (6, 7 y 8). De esta forma, se detalla la metodología referente a la puesta en marcha y aplicación de los sistemas físicos, químicos y biológicos utilizados junto con aquella relativa al seguimiento de dichos sistemas mediante las técnicas analíticas empleadas. Además, este capítulo también incluye el plan de trabajo establecido para la consecución de los objetivos parciales de la tesis.

5.1. Materiales

5.1.1. Paja de arroz

El material lignocelulósico utilizado fue paja de arroz proporcionada por agricultores locales del Parque Natural de la Albufera (Valencia, España; Figura 5.1 a). La paja se secó a temperatura ambiente durante 24 horas, se cortó con tijeras en fragmentos de longitud comprendidos entre 1 y 3 cm y se trituró con una batidora de vaso (Optima 1300, Taurus, España) y, posteriormente, con un molinillo de café eléctrico (ML133, Jata, España). La paja triturada se tamizó con malla metálica de precisión (ISO-3310.1, CISA, España), seleccionando la fracción con un tamaño de partícula comprendido entre 100 y 500 µm, y se secó a 45 °C en estufa con ventilación forzada (Basic A, Memmert, Alemania) hasta reducir la humedad residual a menos del 5% (Figura 5.1 b). Esta fracción de paja se preservó a 4 °C en frigorífico hasta su pretratamiento.

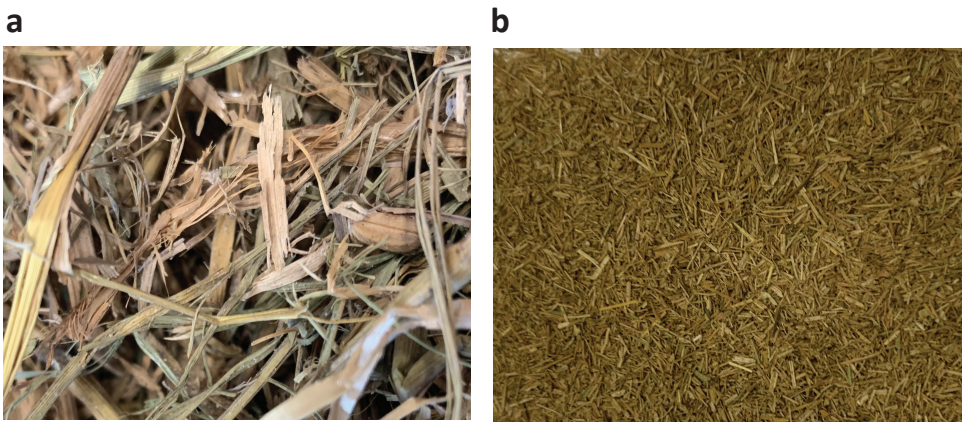

Figura 5.1. Paja de arroz antes (a) y después (b) de cortar, triturar, tamizar y secar.

En el desarrollo de esta tesis doctoral se utilizaron dos lotes de paja de arroz, el lote 1 se empleó en la mayor parte de las experimentaciones y el lote 2 se empleó en el estudio final. La Tabla 5.1 detalla la composición química de cada lote.

Tabla 5.1. Composición química de los lotes de paja de arroz utilizados.

Componente	Composición (% p/p)	
	Lote 1	Lote 2
Glucano	35.8 ± 2.1	35.6 ± 0.6
Xilano	14.8 ± 1.6	17.6 ± 0.5
Arabinano	2.7 ± 0.4	2.1 ± 0.2
Lignina soluble en ácido	0.1 ± 0.0	0.1 ± 0.0
Lignina insoluble en ácido	14.3 ± 0.4	10.4 ± 0.7
Cenizas	16.7 ± 0.1	12.3 ± 0.7

5.1.2. Cepa bacteriana

La cepa *Clostridium beijerinckii* DSM 6422 (NRRL B-592) se adquirió en forma de liófilo de la Colección Alemana de Microorganismos y Cultivos Celulares (*Deutsche Sammelstelle für Mikroorganismen und Zellkulturen*, DSMZ). La recuperación del cultivo liofilizado se llevó a cabo de acuerdo con las instrucciones facilitadas por el suministrador.

La conservación de la cepa bacteriana y la preparación del inóculo se llevaron a cabo en botellas de suero de 50 mL (Supelco, EEUU) en las que el medio empleado se burbujeó con nitrógeno (99.99%; Carburos Metálicos, España) durante 20 minutos con la finalidad de desplazar el oxígeno y asegurar condiciones anaeróbicas, el sistema de burbujeo se muestra en la Figura 5.2 b. Tras desplazar el oxígeno, las botellas se sellaron herméticamente con cierre de aluminio (Fischer Scientific, Dinamarca) y septum de butilo/PTFE (Supelco, EEUU) mediante una crimpadora manual (Supelco, EEUU), esterilizándose a 121 °C durante 21 minutos en un equipo vertical de autoclave (MED20, J.P. Selecta, España). Los cultivos bacterianos se manipularon en una cabina de flujo laminar vertical (AV-100, Telstar, España).

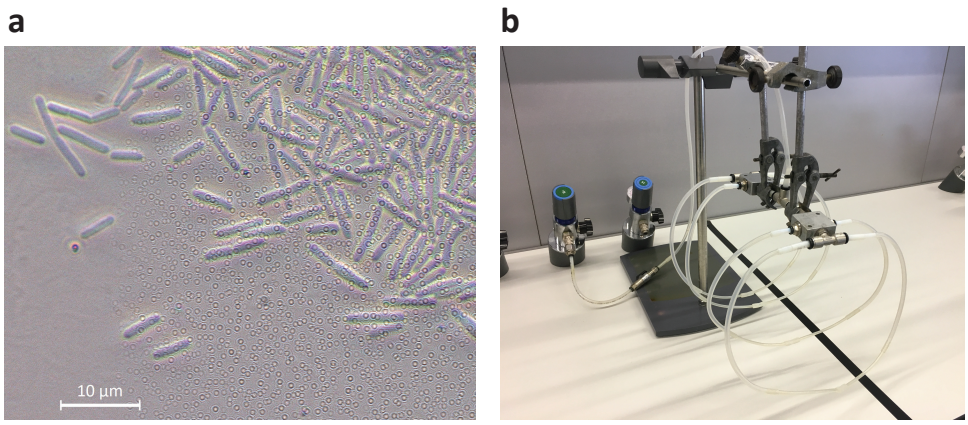

Figura 5.2. Morfología de *C. beijerinckii* DSM 6422 (a, 100x) y sistema de burbujeo (b).

En la conservación de la cepa bacteriana, el cultivo recuperado se inyectó en las botellas con 40 mL de Medio RCM (*Reinforced Clostridial Medium*; VWR, Bélgica) estéril, obteniéndose una relación del 5% (v/v). Las botellas se incubaron a 37 °C durante 24 horas en un equipo estático (INB 200, Memmert, Alemania). El cultivo obtenido se centrifugó a 3000 rpm y 4 °C durante 20 minutos (5804 R, Eppendorf, Alemania), se descartó el sobrenadante y se añadió un volumen de Medio RCM suplementado con glicerol (20% v/v; Fischer Scientific, EEUU) equivalente al doble del volumen de la biomasa producida. La composición de este medio se detalla en la Tabla 5.2. La mezcla obtenida se preservó a -80 °C (ULUF P10, Arctiko, Dinamarca) en criotubos de 2 mL. En la preparación del inóculo para los ensayos de fermentación se inyectaron 50 µl de glicerinado en las botellas con 40 mL de Medio RCM modificado estéril, cuya composición se detalla en la Tabla 5.2. Las botellas se incubaron a 37 °C durante 12 – 24 horas en el equipo estático. Transcurrido ese tiempo, se comprobó que la población bacteriana del inóculo presentaba una densidad óptica a 600 nm (*Optical Density*, OD_{600}) en torno a un valor de 2.

Tabla 5.2. Composición de los medios utilizados en la conservación de la cepa bacteriana y en la preparación del inóculo.

Compuesto	Concentración (g/L)	
	Conservación	Inóculo
Extracto de levadura	2.40	1.50
Extracto de carne	8.00	5.00
Peptona	8.00	5.00
Glucosa	4.00	12.50
Almidón soluble	0.80	0.50
Cloruro de sodio	4.00	2.50
Acetato de sodio	2.40	1.50
L-Cisteína clorhidrato	0.40	0.25
Agar	0.40	0.25
Glicerol	252.20	-

5.1.3. Enzimas

La hidrólisis enzimática se llevó a cabo empleando la mezcla de enzimas Cellic® CTec2 suministrada por la empresa Novozymes (Dinamarca). Cellic® CTec2 es un preparado líquido de celulasas y hemicelulasas producido por el hongo *Trichoderma reesei* y ampliamente utilizado en estudios sobre hidrólisis de biomasa lignocelulósica. Esta mezcla de enzimas se seleccionó para el desarrollo de esta tesis doctoral, debido a que en comparación con otros preparados comerciales de

celulasas presenta los mayores niveles de actividad celobiohidrolasa, xilanasa y β-glucosidasa (Yang y col., 2017) y en comparación con preparados comerciales de hemicelulasas presenta relativamente una gran actividad xilanasa (Aramrueang y col., 2017). A lo largo de la tesis se utilizaron tres lotes de Cellic® CTec2 con una actividad comprendida entre 119 y 193 unidades de papel de filtro (*Filter Paper Unit*, FPU) por mililitro de mezcla.

5.2. Métodos

5.2.1. Pretratamiento de la paja de arroz

La paja de arroz se pretrató mediante dos métodos: hidrotermólisis asistida por microondas (método físico) y pretratamiento alcalino con hidróxido sódico (método químico). A modo de ejemplo, la Figura 5.3 muestra la paja pretratada con ambos métodos.

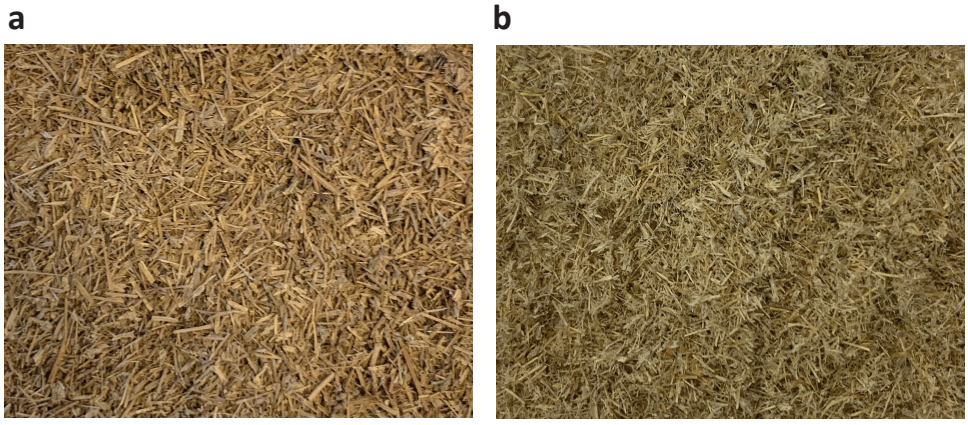

Figura 5.3. Paja de arroz pretratada con microondas (a) y con hidróxido sódico (b).

5.2.1.1. Hidrotermólisis asistida por microondas

El pretratamiento hidrotérmico se llevó a cabo en un sistema de digestión por microondas (Ethos 1, Milestone, Italia) con control directo de temperatura por termopar (ATC-400CE, Milestone, Italia) y con un rotor segmentado de alta presión (HPR-1000/10S, Milestone, Italia) que permite trabajar con hasta 10 vasos de TFM (*modified polytetrafluoroethylene*) de 100 mL de capacidad. La temperatura en el digestor se controló mediante la variación de la intensidad emitida (máxima de 1500 W). En cada vaso se introdujo la paja de arroz seca y se mezcló con agua desionizada hasta obtener una carga de sólidos del 10% (p/v) en un volumen final de 30 mL. La mezcla se sometió al programa de temperaturas presentado en la Figura 5.4. Tras aplicar 200 °C durante 15 minutos, el sistema se dejó enfriar a temperatura

ambiente. Una vez finalizado el pretratamiento, el contenido de los vasos se centrifugó a 10000 rpm durante 5 minutos (5804, Eppendorf, Alemania). La fase sólida recuperada se lavó en dos ciclos con agua desionizada, incluyendo un control de pH a 6.5 en el segundo ciclo. El sólido pretratado se secó a 45 °C durante 48 horas en la estufa y se preservó a 4 °C hasta su uso.

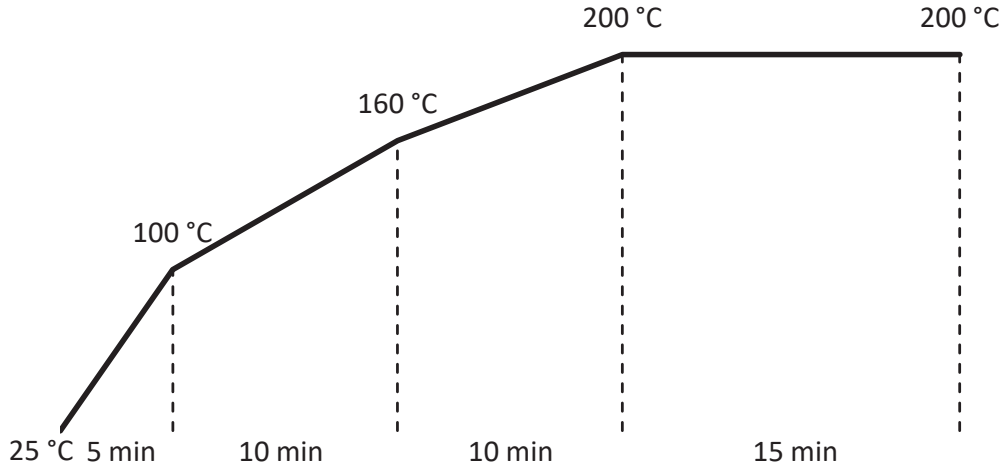

Figura 5.4. Programa de temperaturas del digestor en el pretratamiento de la paja de arroz con microondas.

5.2.1.2. Pretratamiento alcalino con hidróxido sódico

En el pretratamiento alcalino, la paja de arroz seca se mezcló con disoluciones de hidróxido sódico con concentraciones de hasta el 2% (p/v; VWR, República Checa) en botellas de vidrio de 1 litro (Simax, República Checa), trabajando con cargas de sólidos de hasta el 10% (p/v) en un volumen final de 720 mL. Las botellas se calentaron durante un tiempo máximo de 60 minutos en el equipo de autoclave que permite trabajar a dos niveles de temperatura (121 y 134 °C) y el sistema se dejó enfriar a temperatura ambiente tras el calentamiento. Una vez finalizado el pretratamiento, el contenido de las botellas se centrifugó a 4000 rpm durante 6 minutos (Mega Star 3.0, VWR, Alemania). La fase sólida recuperada se lavó en cuatro ciclos con agua desionizada, incluyendo un control de pH a 6.5 en el último ciclo. El sólido pretratado se secó a 45 °C durante 48 horas en la estufa y se preservó a 4 °C hasta su uso.

5.2.2. Hidrólisis enzimática de la paja de arroz pretratada

La hidrólisis enzimática de la paja de arroz pretratada se llevó a cabo en matraces Erlenmeyer de 100 mL con tapón de rosca (Schott Duran, Alemania) en los que se introdujo la paja y se mezcló con una disolución de tampón (pH 5.0),

trabajando con cargas de sólidos de hasta el 10% (p/v) en un volumen final de 50 mL. Tras añadir la mezcla Cellic® CTec2 con cargas de hasta 15 FPU por gramo de biomasa pretratada, los matraces permanecieron a 50 °C y 150 rpm durante 72 horas en un agitador orbital (SI500, Stuart, RU). Una vez finalizada la hidrólisis, el hidrolizado se centrifugó a 4000 rpm durante 6 minutos (5804, Eppendorf, Alemania) y el sobrenadante se filtró por 1.2 µm y se preservó a 4 °C hasta su uso.

5.2.3. Fermentación ABE

5.2.3.1. Fermentación ABE por SHF

En el proceso SHF, el hidrolizado obtenido en la hidrólisis enzimática se fermentó en botellas de suero de 50 mL a las que se acoplaron válvulas de tres vías estériles (Discofix® C, B. Braun, Suiza) para la toma de muestras (Figura 5.5 a). El hidrolizado se suplementó en base a la modificación del Medio P2 desarrollado por Monot y col. (1982), cuya composición se detalla en la Tabla 5.3. Se utilizó resazurina sódica, indicador redox que no inhibe el crecimiento de los microorganismos, para detectar la presencia de oxígeno en el medio.

Tabla 5.3. Composición del Medio P2 modificado.

Compuesto	Concentración
Extracto de levadura	A optimizar
Acetato amónico	2.20 g/L
Fosfato monopotásico	0.50 g/L
Fosfato dipotásico	0.50 g/L
Sulfato de magnesio heptahidratado	0.09 g/L
Sulfato de manganeso (II) monohidratado	1 mg/L
Sulfato de hierro (II) heptahidratado	A optimizar
Resazurina sódica	1 mg/L

En las botellas se introdujo el hidrolizado, la resazurina y una disolución con extracto de levadura y compuestos tampón (acetato amónico y especies de potasio hidrogenofosfato) previamente autoclavada a 121 °C durante 21 minutos. Tras ajustar el pH en valores de hasta 7.4, se desplazó el oxígeno de igual forma que en la Sección 5.1.2 y las botellas se autoclavaron a 121 °C durante 10 minutos. Cuando el sistema se enfrió a temperatura ambiente, se inyectó la disolución de minerales (sulfato de magnesio, sulfato de manganeso y sulfato de hierro), previamente esterilizada mediante filtración por 0.2 µm, y el inóculo con una OD_{600} de

aproximadamente 2 en una relación del 5% (v/v), obteniéndose volúmenes finales de hasta 40 mL. Las botellas se incubaron en el agitador orbital a 37 °C y 150 rpm durante el tiempo establecido en cada ensayo.

5.2.3.2. Fermentación ABE por SSF

En el proceso SSF, la paja de arroz pretratada se hidrolizó y fermentó simultáneamente en la misma botella de suero de 50 mL, suplementándose el medio de igual forma que en la Sección 5.2.3.1. En las botellas se introdujo la paja pretratada seca, trabajando con cargas finales de sólidos de hasta el 12.2% (p/v), la resazurina y la disolución estéril con extracto de levadura y compuestos tampón. En este caso, el volumen relativo al hidrolizado se sustituyó por agua desionizada. Tras ajustar el pH en valores de hasta 7.4, se desplazó el oxígeno y las botellas se autoclavaron a 121 °C durante 10 minutos. Cuando el sistema se enfrió a temperatura ambiente, se inyectó la disolución estéril de minerales, la mezcla Cellic® CTec2 con cargas de hasta 26.3 FPU/g y el inóculo con una OD_{600} de aproximadamente 2 en una relación del 5% (v/v), obteniéndose volúmenes finales de hasta 40 mL. Las botellas se incubaron en el agitador orbital a 37 °C y 150 rpm durante el tiempo establecido en cada ensayo.

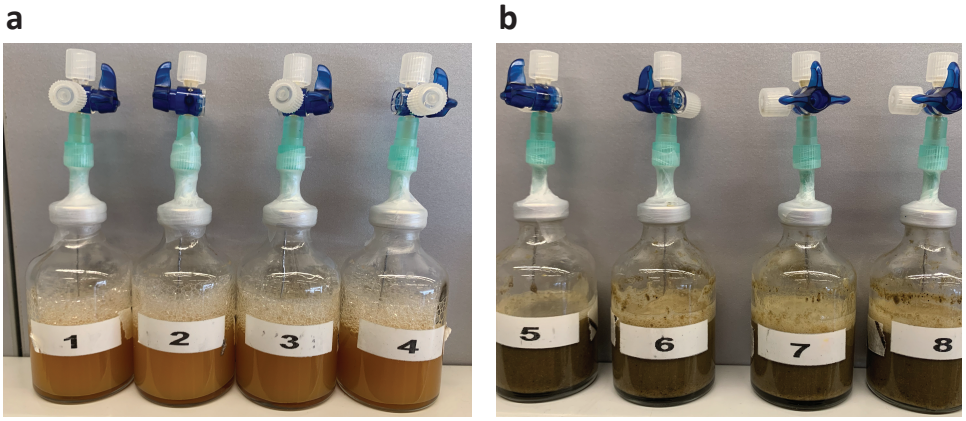

Figura 5.5. Fermentación de hidrolizados de paja de arroz pretratada con hidróxido sódico (a) y SSF de paja de arroz pretratada con hidróxido sódico (b).

5.2.3.3. Fermentación ABE por SSFR en modo semicontinuo

Como configuración final se aplicó el proceso SSF en modo de alimentación semicontinuo incluyendo recuperación *in situ* de producto, empleando un reactor de tanque agitado (*Stirred Tank Reactor*, STR) de 2 litros fabricado mediante la modificación de una botella de vidrio (Schott Duran, Alemania; Figura 5.7). La Figura 5.6 muestra el esquema del montaje experimental. La temperatura del reactor se

mantuvo en 37 °C con un baño calefactor (WNB 10, Memmert, Alemania) y el volumen de trabajo se mantuvo en 500 mL mediante la adición intermitente de agua destilada estéril con una bomba peristáltica Ismatec® (Cole-Parmer, EEUU). La operación unitaria utilizada en la recuperación *in situ* de producto fue el arrastre por gas, los gases producidos en la fermentación (dióxido de carbono e hidrógeno) se recircularon y se hicieron burbujear en el caldo de fermentación mediante una bomba de vacío (VP 86, VWR, Francia) a un caudal volumétrico de 4 L/min. Los disolventes producidos en la fermentación (acetona y butanol) y transferidos a la corriente gaseosa se recuperaron en un condensador de bolas (200 mm longitud x 115 cm^2 área de superficie de enfriamiento: Vidrafoc, España) que se mantuvo a 4 °C con un baño de circulación refrigerado (AD15R-30, VWR, EEUU). El condensado se transfirió periódicamente a un recipiente colector para la determinación de su volumen y composición.

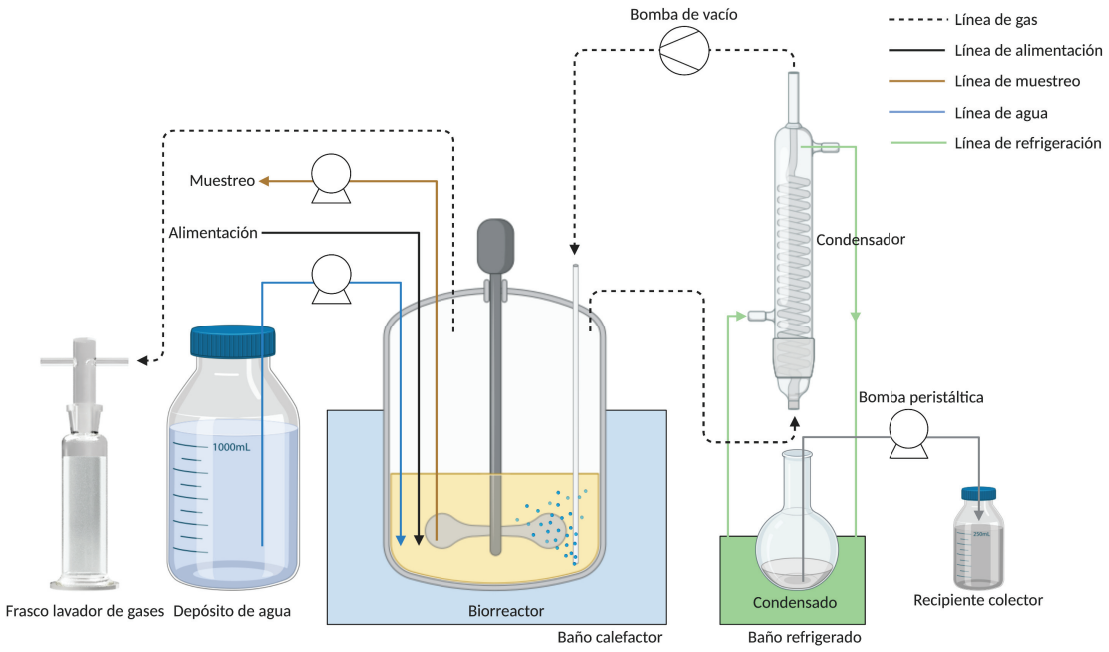

Figura 5.6. Esquema del montaje experimental empleado en el proceso SSFR en modo semicontinuo. Creado con BioRender.com.

La puesta en marcha y las condiciones de operación fueron las mismas que las descritas en la Sección 5.2.3.2, salvo que el oxígeno se desplazó tras esterilizar y que la agitación se redujo a 120 rpm. El nitrógeno burbujeado se esterilizó a través de un filtro Millex® (0.2 µm; Millipore, Francia). La paja de arroz pretratada se añadió en tres adiciones: un 50% de la cantidad total al inicio del proceso, un 25% tras 20 horas de fermentación en modo discontinuo (antes de que la glucosa fuese completamente consumida) y el 25% restante tras 30 horas de operación (para asegurar la solubilización del sólido previamente agregado), resultando en una

carga final de sólidos del 18.4% (p/v). En la alimentación de sustrato, se hizo circular nitrógeno estéril (filtrado por 0.2 µm) por el espacio de cabeza del reactor para asegurar las condiciones anaeróbicas. El arrastre por gas se encendió a las 20 horas y se apagó a las 50 horas para mantener una concentración de butanol en el medio de fermentación comprendida entre 5 y 12 g/L.

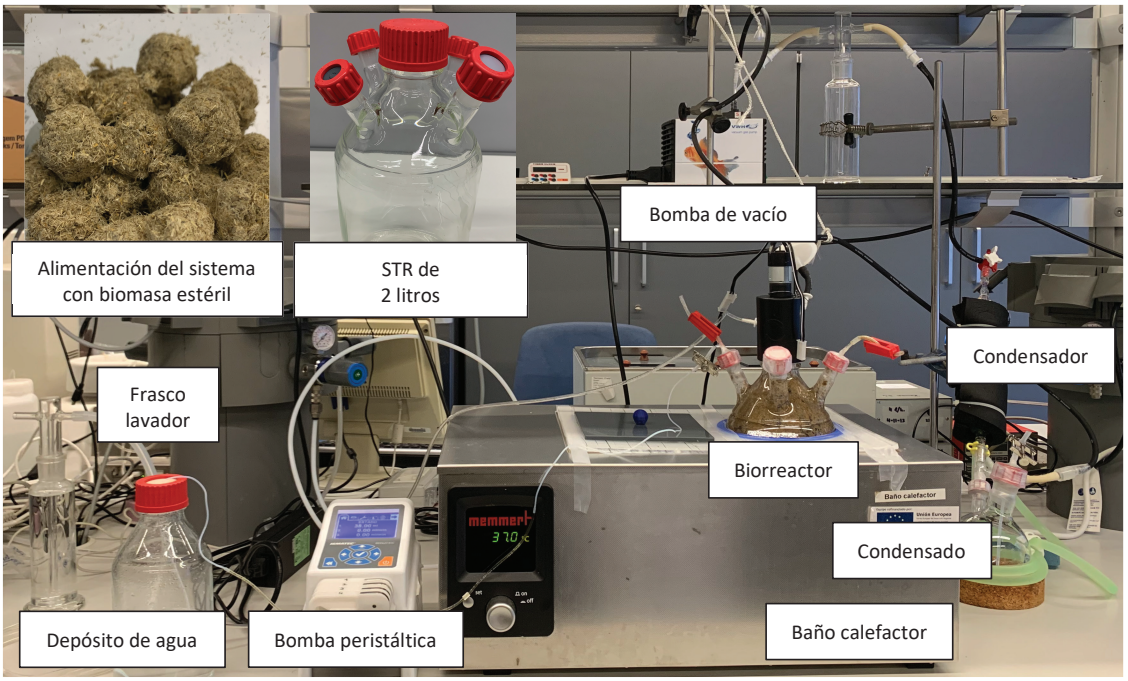

Figura 5.7. Imagen del proceso SSFR en modo semicontinuo a partir de paja de arroz pretratada con hidróxido sódico.

5.2.4. Técnicas analíticas

Los métodos desarrollados por el Laboratorio Nacional de Energía Renovable (*National Renewable Energy Laboratory*, NREL) se utilizaron para determinar el contenido en humedad (TP-510-42621, Sluiter y col., 2008a) y en carbohidratos, lignina y cenizas (TP-510-42618, Sluiter y col. 2008b) de la paja de arroz y, además, para determinar la actividad enzimática de la mezcla Cellic® CTec2 (TP-510-42628, Adney y Baker, 1996).

Los hidrolizados obtenidos se caracterizaron a partir de muestras de 1.5 mL, analizando la concentración de los monosacáridos liberados (arabinosa, glucosa y xilosa) y de los compuestos inhibidores que podían producirse en el pretratamiento (ácido acético, ácido levulínico, HMF y furfural). El seguimiento de las fermentaciones se realizó a partir de muestras de 1 – 2 mL, analizando el pH (Minitrode, Hamilton, EEUU), la densidad celular, la concentración de monosacáridos presentes en el caldo de cultivo y la concentración de los ácidos

(acético y butírico) y los disolventes (acetona y butanol) producidos. La concentración de monosacáridos se determinó mediante cromatografía iónica (*Ion Chromatography*, IC) o cromatografía líquida de alta eficacia (*High Performance Liquid Chromatography*, HPLC), la concentración de inhibidores se determinó mediante HPLC y la concentración de ácidos y disolventes se determinó mediante cromatografía de gases (*Gas Chromatography*, GC) o HPLC. Como paso previo al análisis de las muestras por cromatografía estas se centrifugaron a 10000 rpm durante 5 minutos (Micro Star 12, VWR, Corea) y el sobrenadante se filtró por 0.22 µm. Los reactivos con alto grado de pureza utilizados en la elaboración de los calibrados para las técnicas cromatográficas fueron suministrados por Acros Organics, Merck, VWR, Alfa Aesar y Sigma-Aldrich. La concentración de los compuestos fenólicos totales, un tipo de inhibidores originados a partir de la lignina, se determinó mediante el método desarrollado por Folin y Denis (1912) y se expresó como equivalentes de ácido gálico (*Gallic Acid Equivalents*, GAE).

5.2.4.1. Determinación de la densidad celular

La densidad celular se cuantificó a partir de la OD_{600} analizada con un espectrofotómetro (SpectroFlex 6600, WTW, EEUU). Cada muestra se analizó con tres mediciones repetidas. Previamente, se correlacionó la OD_{600} con la densidad celular de la cepa *C. beijerinckii* DSM 6422 mediante 10 muestras en las que la masa seca se determinó en base a los sólidos totales de acuerdo con el método del NREL (TP-510-42621, Sluiter y col., 2008a). La Figura 5.8 presenta la recta de calibrado obtenida junto con la ecuación lineal de regresión y el coeficiente de correlación.

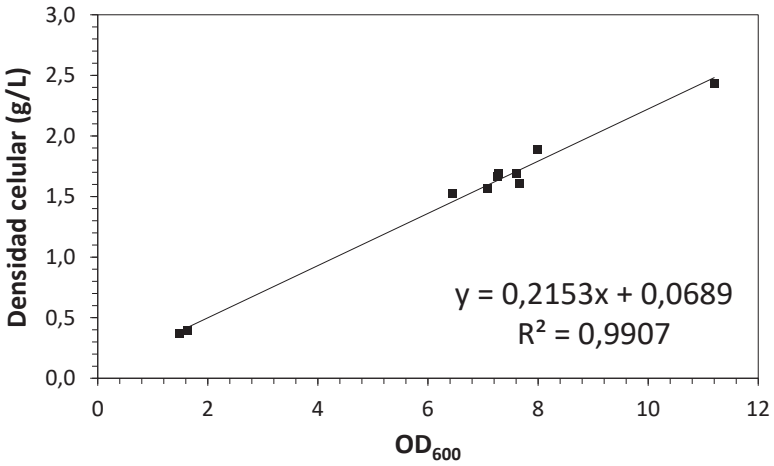

Figura 5.8. Recta de calibrado para la determinación de la densidad celular de *C. beijerinckii* DSM 6422.

En los ensayos SSF en modo discontinuo y SSFR en modo semicontinuo, la densidad celular no se pudo cuantificar debido a las interferencias asociadas a la paja de arroz presente en el medio.

5.2.4.2. Determinación de la concentración de monosacáridos por cromatografía iónica

En el estudio comparativo de los procesos SSF y SHF, la concentración de los monosacáridos se determinó mediante un cromatógrafo iónico (883 Basic IC plus, Metrohm, Suiza) equipado con una columna Metrosep Carb 2-150/4.0 (Metrohm, Suiza) de 150 mm de longitud, un diámetro interno de 4 mm y un tamaño de partícula de 5 µm. El equipo dispone de un detector amperométrico (945 Professional Detector Vario, Metrohm, Suiza). Como fase móvil se utilizó una disolución de hidróxido sódico 20 mM a un caudal de 0.5 mL/min. La calibración del cromatógrafo iónico se realizó para cada conjunto de análisis, empleando multipatrones de monosacáridos a partir de los cuales se estableció un intervalo de concentraciones adecuado a las condiciones de trabajo. En la Figura 5.9 se muestra la distribución de los picos de cada compuesto.

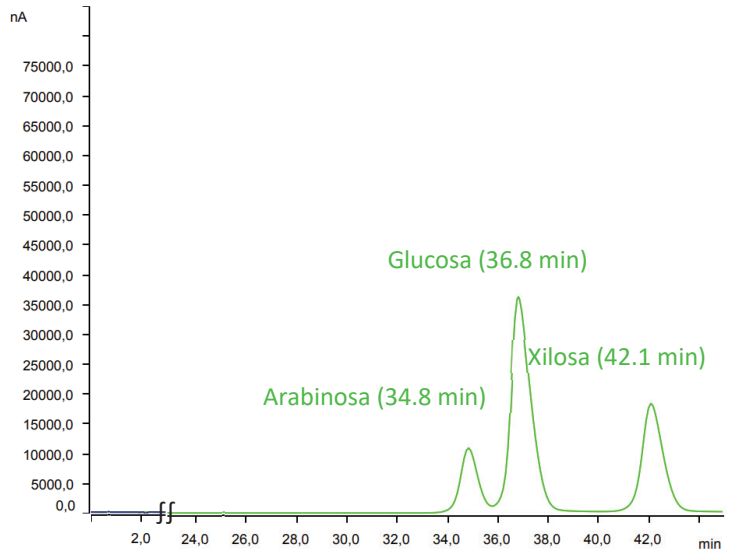

Figura 5.9. Cromatograma de los patrones en IC.

5.2.4.3. Determinación de la concentración de ácidos y disolventes por cromatografía de gases

En el estudio comparativo de los procesos SSF y SHF, la concentración de los ácidos y disolventes se determinó mediante un cromatógrafo de gases (TRACE GC Ultra, Thermo Scientific, Italia) equipado con una columna TRB-FFAP (Teknokroma, España) de 30 metros de longitud, un diámetro interno de 0.25 mm y un espesor de

película de 0.25 µm. El equipo dispone de un inyector automático (AI 3000, Thermo Scientific, Italia) y un detector de ionización de llama. Como gas portador se utilizó helio (99.99%; Carburos Metálicos, España). Las condiciones cromatográficas de análisis se detallan en la Tabla 5.4.

Tabla 5.4. Parámetros del método utilizado en GC.

Volumen de inyección	1 µL
Temperatura del inyector	250 °C
"Split ratio" en inyección	10:1
Caudal de gas portador (helio)	1.0 mL/min
Temperatura del detector	250 °C

El programa de temperaturas en el horno se presenta en la Figura 5.10. La calibración del cromatógrafo de gases se realizó periódicamente, empleando multipatrones de ácidos y disolventes a partir de los cuales se estableció un intervalo de concentraciones adecuado a las condiciones de trabajo. La bondad de la calibración se comprobó cada 15 días. Las muestras y los patrones se acidificaron a un pH menor a 3 mediante una disolución de ácido fosfórico al 17% (v/v), de acuerdo con Boušková y col. (2005), para evitar la ionización de los ácidos. Cada muestra se analizó con, al menos, dos mediciones repetidas.

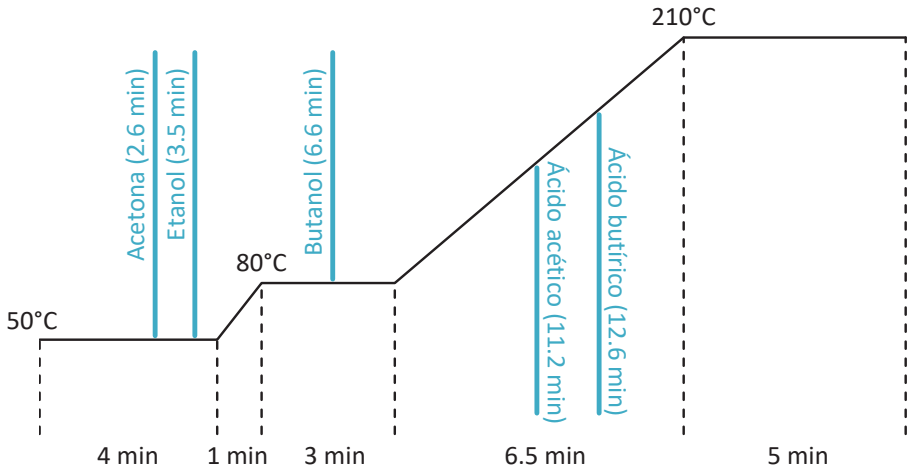

Figura 5.10. Programa de temperaturas en GC y tiempos de retención de los patrones.

5.2.4.4. Determinación de la concentración de monosacáridos, ácidos, disolventes e inhibidores por HPLC

En la evaluación del pretratamiento alcalino de la paja de arroz y en el desarrollo del proceso SSFR en modo de alimentación semicontinuo, la concentración de los monosacáridos, ácidos, disolventes y compuestos inhibidores

se determinó mediante un cromatógrafo de líquidos de alta resolución (Agilent HPLC 1100 Series, Agilent Technologies, Alemania) equipado con una columna Aminex® HPX-87H (Bio-Rad Laboratories Inc., EEUU) de 300 mm de longitud y un diámetro interno de 7.8 mm. El equipo dispone de un detector de índice de refracción (*Refractive Index Detector*, RID) y un detector ultravioleta de fotodiodos (*Diode Array Detector*, DAD) que operó a dos longitudes de onda (210 y 280 nm). Como fase móvil se utilizó una disolución de ácido sulfúrico 5 mM. Las condiciones cromatográficas de análisis se detallan en la Tabla 5.5.

Tabla 5.5. Parámetros del método utilizado en HPLC.

Volumen de inyección	5 µL
Caudal de fase móvil en columna	0.6 mL/min
Temperatura de columna	50 °C
Temperatura del detector RID	35 °C

En la Tabla 5.6 se muestra el tipo de detector por el que se analizó cada compuesto y los respectivos tiempos de retención.

Tabla 5.6. Detección de los compuestos en HPLC.

Compuesto	**Detector**	**Tiempo de retención (min.)**
Ácido levulínico	$DAD_{210\ nm}$	15.9
Ácido butírico	$DAD_{210\ nm}$	21.4
Acetona	$DAD_{280\ nm}$	22.3
HMF	$DAD_{280\ nm}$	31.9
Furfural	$DAD_{280\ nm}$	48.9
Glucosa	RID	8.8
Xilosa	RID	9.5
Arabinosa	RID	10.3
Ácido acético	RID	15.1
Etanol	RID	21.5
Butanol	RID	38.1

La calibración del HPLC se realizó periódicamente, empleando multipatrones de monosacáridos, ácidos, disolventes y compuestos inhibidores a partir de los cuales se estableció un intervalo de concentraciones adecuado a las condiciones de trabajo. La bondad de la calibración se comprobó cada 15 días.

5.2.4.5. Microscopía electrónica de barrido

La adsorción de las células bacterianas a la superficie de la paja de arroz pretratada se observó mediante el equipo de microscopía electrónica de barrido (*Scanning Electron Microscope*, SEM) S-4800 (Hitachi, Japón), perteneciente al Servei Central de Suport a la Investigació Experimental de la Universitat de València. Este equipo emplea un voltaje de aceleración de 20 kV. Como paso previo a la observación en el SEM las muestras se deshidrataron en disoluciones alcohólicas seriadas. Posteriormente, se secaron en el punto crítico con CO_2, se fijaron en una placa metálica y, finalmente, se recubrieron de oro y paladio en atmósfera inerte de argón.

5.2.5. Diseño de experimentos

El diseño de experimentos (*Design of Experiments*, DOE) se define como: *"el proceso para planear el experimento de tal forma que se recaben datos adecuados que puedan analizarse con métodos estadísticos que llevarán a conclusiones válidas y objetivas"* (Montgomery, 2016a). La aplicación de esta herramienta suele incluir dos partes. La primera parte es la identificación de los factores críticos del proceso, aquellas variables de entrada con mayor influencia sobre la variable respuesta, mediante técnicas de cribado como los diseños factoriales fraccionados (*Fractional Factorial Design*, FFD). La segunda parte es la optimización del proceso mediante metodología de superficie de respuesta (*Response Surface Methodology*, RSM). Una técnica de RSM son los diseños centrales compuestos (*Central Composite Design*, CCD), los cuales permiten obtener los niveles de los factores que maximizan la variable respuesta. En el desarrollo de esta tesis doctoral, se utilizó el programa estadístico MINITAB® v.2020.1.0 (Minitab Inc., EEUU) para el DOE y para el posterior análisis estadístico de los datos.

5.2.5.1. Diseños factoriales fraccionados 2^{4-1} (resolución IV)

Los FFD son transformaciones de los diseños factoriales básicos en las que únicamente se efectúa un subconjunto de los ensayos al considerar despreciables ciertas interacciones entre los factores estudiados, de forma que no se realizan todas las posibles combinaciones de niveles. Un diseño 2^{4-1} es la fracción un medio de un diseño 2^4 en la que se estudian cuatro factores, cada uno con dos niveles, a partir de ocho ensayos. En los FFD de resolución IV, los efectos principales se pueden diferenciar entre sí y de las interacciones entre dos factores, pero los efectos de las interacciones entre dos factores no se pueden diferenciar entre sí (Montgomery,

2016b). En la Figura 5.11 se muestran las gráficas de cubo típicas de los diseños 2^{4-1} con 8 ensayos utilizados en esta tesis doctoral.

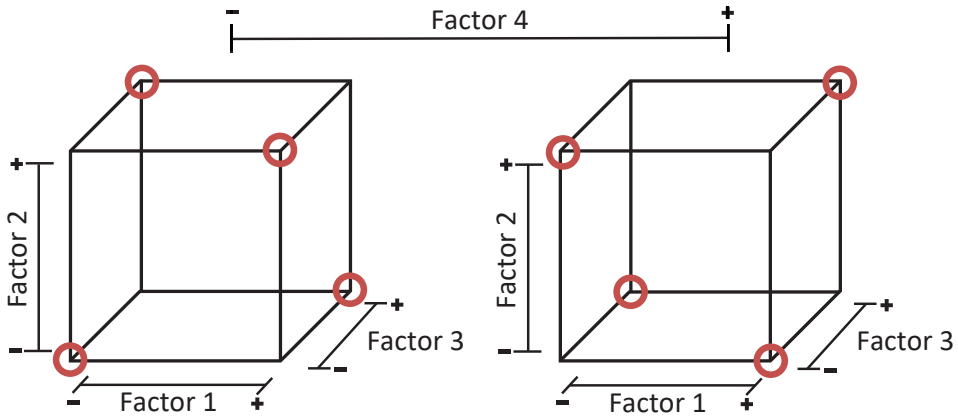

Figura 5.11. Gráficas de cubo del FFD 2^{4-1} (resolución IV). Los vértices de los cubos indican los niveles bajos (-) y altos (+) de los factores 1, 2 y 3. El cubo de la izquierda representa el factor 4 en su nivel bajo (-) y el de la derecha el factor 4 en su nivel alto (+). Los círculos rojos indican las combinaciones evaluadas en los ensayos. Adaptada de Box y col. (2008).

5.2.5.2. Diseños centrales compuestos

Los CCD son el tipo de diseño más común entre los métodos RSM. Está técnica permite obtener un modelo de segundo orden si el sistema presenta curvatura. Para ello incorpora los ensayos con niveles axiales con una distancia α al centro del diseño. El número de ensayos axiales es $2 \times k$, siendo k el número de factores. Los diseños CCD presentan también un determinado número de ensayos con niveles centrales que permiten determinar la varianza de la respuesta predicha. Para que el diseño sea rotable, es decir, que todos los puntos que están a la misma distancia del centro del diseño presenten la misma varianza de la respuesta predicha, α debe de ser igual a $n_F^{1/4}$, siendo n_F el número de puntos de la sección factorial del diseño (Montgomery, 2016c). La siguiente ecuación muestra un modelo de regresión lineal de segundo orden obtenido tras el ajuste de los datos derivados del CCD:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \times x_i + \sum_{i=1}^{k} \beta_{ii} \times x_i^2 + \sum \sum_{i<j} \beta_{ij} \times x_i \times x_j + \varepsilon$$

Siendo y la respuesta predicha, β_0 la intersección del plano con el eje de coordenadas, k el número de variables independientes, x_i y x_j los valores de las variables independientes, β_i el coeficiente de regresión parcial linear, β_{ii} el

coeficiente de regresión parcial cuadrático, β_{ij} el coeficiente de regresión parcial de la interacción entre variables independientes y ε el error de la respuesta.

Los CCD utilizados en esta tesis doctoral están compuestos por un diseño factorial 2^2, 4 ensayos axiales y 5 ensayos centrales (13 ensayos) o por un diseño factorial 2^3, 6 ensayos axiales y 6 ensayos centrales (20 ensayos). En la Figura 5.12 se muestran las gráficas características de ambos diseños.

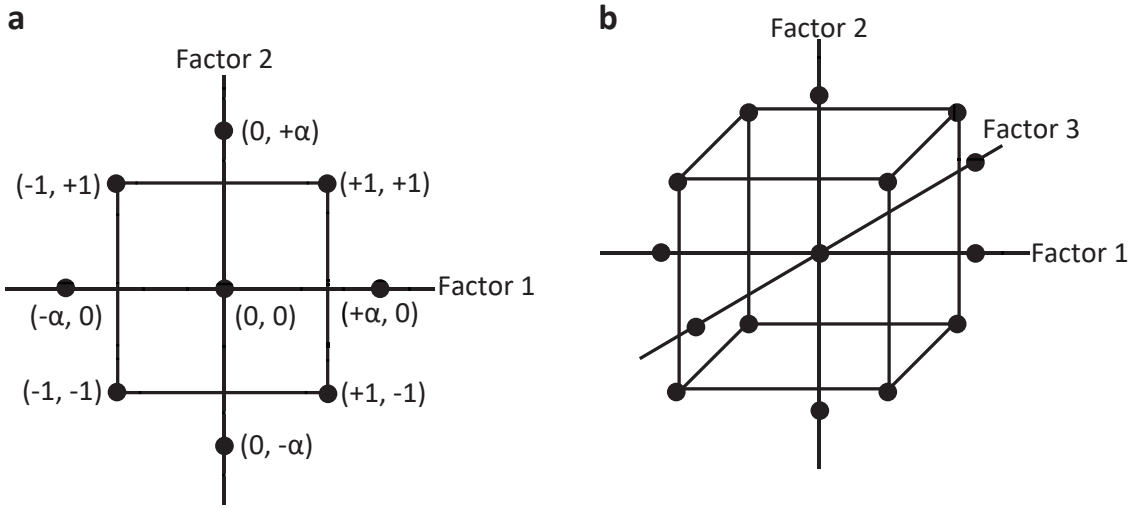

Figura 5.12. Gráficas del CCD con dos (a) y tres (b) factores. Los puntos de los vértices del cuadrado y del cubo se corresponden con los ensayos del diseño factorial, los puntos externos se corresponden con los ensayos axiales y los puntos internos se corresponden con los ensayos centrales. Adaptada de Montgomery (2016c).

5.3. Plan de trabajo

A continuación, se describe el plan de trabajo que se siguió para la consecución de los objetivos parciales de la tesis:

- Estudio comparativo de los procesos SSF y SHF.
- Evaluación del pretratamiento alcalino de la paja de arroz.
- Desarrollo del proceso SSFR en modo de alimentación semicontinuo.

5.3.1. Estudio comparativo de los procesos SSF y SHF

El estudio comparativo de los procesos SSF y SHF se llevó a cabo utilizando como sustrato paja de arroz pretratada mediante hidrotermólisis asistida por microondas. Mediante dos FFD 2^{4-1} (resolución IV) se analizó en ambas configuraciones el efecto del pH inicial y de la concentración de dos componentes

(extracto de levadura y sulfato de hierro) en el medio de fermentación sobre la producción de biobutanol. Adicionalmente, en el proceso SHF se estudió el efecto del tampón utilizado en la hidrólisis enzimática y en el proceso SSF se estudió el efecto de la carga enzimática. La Tabla 5.7 detalla los valores codificados y reales de todas las variables independientes en los FFD. Los niveles de los componentes del medio estudiados y la concentración del resto de componentes fijados (Tabla 5.3) se establecieron en experimentos previos realizados en el equipo de investigación en los que se utilizaron mezclas de glucosa y xilosa (proporción G:X = 2:1), con una concentración total de azúcares de 20 y 60 g/L, como fuente de carbono.

Tabla 5.7. FFD 2^{4-1} (resolución IV) para los procesos SHF y SSF a partir de paja de arroz pretratada con microondas.

Proceso SHF			
Variables independientes		**Valores codificados y reales**	
		Nivel -1	**Nivel +1**
X_1	Extracto de levadura (g/L)	2.00	4.00
X_2	Sulfato de hierro (g/L)	0.01	0.02
X_3	pH inicial	6.4	7.4
X_4	Tampón en hidrólisis[a]	Acetato 50 mM	Citrato 50 mM
Proceso SSF			
Variables independientes		**Valores codificados y reales**	
		Nivel -1	**Nivel +1**
X_1	Extracto de levadura (g/L)	2.00	4.00
X_2	Sulfato de hierro (g/L)	0.01	0.02
X_3	pH inicial	5.2	6.2
X_4	Carga enzimática (FPU/g)	4.1	12.4

[a] Categórica.

La concentración de butanol producido a las 48 horas se seleccionó como variable respuesta, ya que solamente se observó un incremento menor al 1% en la concentración de disolventes tras ese tiempo. La Tabla 5.8 resume las condiciones de operación fijadas en ambos procesos.

Tabla 5.8. Condiciones de operación fijadas en los procesos SHF y SSF a partir de paja de arroz pretratada con microondas.

Proceso SHF		
Hidrólisis enzimática	Carga de sólidos (% p/v)	10.0
	Carga enzimática (FPU/g)	4.1
Proceso SSF		
Hidrólisis enzimática + fermentación ABE	Carga de sólidos (% p/v)	9.0

Para determinar el rendimiento de la hidrólisis enzimática en el proceso SSF se llevó a cabo un experimento control por duplicado, empleando el mismo medio de fermentación y la misma carga de sólidos, pero sin inoculación. Para este experimento control, se seleccionó el mínimo pH (5.2) y la máxima carga enzimática (12.4 FPU/g) por ser estas las condiciones más favorables para la hidrólisis.

A partir de los resultados obtenidos en el estudio comparativo, se estableció el proceso SSF como la configuración más eficiente y se completó el primer estudio de la tesis doctoral con la optimización de dicho proceso mediante un CCD. Este diseño estadístico se aplicó para obtener el modelo de regresión de segundo orden de la producción de butanol a las 48 horas en función de las variables de estudio: carga enzimática, pH inicial y concentración de extracto de levadura. La Tabla 5.9 detalla los valores codificados y reales de las tres variables independientes estudiadas.

Tabla 5.9. CCD de 3 variables para el proceso SSF a partir de paja de arroz pretratada con microondas. α = 1.68.

	Variables independientes	**Valores codificados y reales**				
		Nivel -α	**Nivel -1**	**Punto central (0)**	**Nivel +1**	**Nivel +α**
Z_1	Carga enzimática (FPU/g)	5.4	8.2	12.2	16.3	19.1
Z_2	pH inicial	5.6	5.9	6.4	6.9	7.2
Z_3	Extracto de levadura (g/L)	0.50	1.50	3.00	4.50	5.50

La carga de sólidos se mantuvo en 9% (p/v) y, en base a los resultados del FFD preliminar, se estableció en estos ensayos y en el resto de los ensayos de la tesis doctoral una concentración de sulfato de hierro en el medio de fermentación de 0.02 g/L. En este caso, el rendimiento de la hidrólisis enzimática se determinó mediante un experimento control sin inoculación en el que se seleccionó una carga enzimática de 12.2 FPU/g, un pH inicial de 6.4 y una concentración de extracto de levadura de 3 g/L. Para comprobar la capacidad de predicción del modelo de regresión obtenido se llevó a cabo un experimento de validación por triplicado, utilizando los valores de las variables independientes que maximizaban la producción de butanol a las 48 horas.

5.3.2. Evaluación del pretratamiento alcalino de la paja de arroz

Los resultados del primer estudio de la tesis doctoral indicaron que la celulosa y la hemicelulosa de la paja de arroz pretratada mediante hidrotermólisis asistida por microondas presentaban una accesibilidad reducida a las enzimas hidrolíticas. De esta forma, se evaluó el pretratamiento alcalino con hidróxido sódico como

método prometedor en referencia a la deslignificación de la paja de arroz. En este segundo estudio se empleó la configuración SHF para analizar los efectos de las variables del pretratamiento sobre la hidrólisis enzimática y la fermentación de forma individual. Como etapa previa se determinaron las variables independientes con efecto significativo sobre la conversión de biomasa en biobutanol y, posteriormente, se optimizó el pretratamiento. En ambos casos, se utilizó como variable respuesta la relación butanol-biomasa obtenida al final de la fermentación (72 horas). Esta variable permite cuantificar la conversión de biomasa en biobutanol al considerar el balance de masas de las tres etapas del proceso (pretratamiento, hidrólisis y fermentación).

Mediante un FFD 2^{4-1} (resolución IV) se analizó el efecto de la temperatura de reacción, del tiempo de reacción, de la concentración de hidróxido sódico y de la carga de sólidos sobre la relación butanol-biomasa a las 72 horas. La Tabla 5.10 detalla los valores codificados y reales de las variables independientes en el FFD.

Tabla 5.10. FFD 2^{4-1} (resolución IV) para el pretratamiento alcalino de la paja de arroz.

Variables independientes		Valores codificados y reales	
		Nivel -1	**Nivel +1**
X_1	Temperatura (°C)[a]	121	134
X_2	Tiempo (min)	10	40
X_3	Concentración NaOH (% p/v)	0.20	2.00
X_4	Carga de sólidos (% p/v)[a]	5.0	10.0

[a] Categórica.

La hidrólisis enzimática y la fermentación se llevaron a cabo mediante las condiciones resumidas en la Tabla 5.11. En base a los resultados del primer estudio, se estableció en estos ensayos y en el resto de los ensayos de la tesis doctoral el uso del tampón acetato 50 mM en la etapa separada de hidrólisis y una concentración de extracto de levadura en el medio de fermentación de 4 g/L. Los resultados de experimentos previos realizados en el equipo de investigación determinaron el resto de las condiciones de operación de la hidrólisis enzimática.

Tabla 5.11. Condiciones de operación en el proceso SHF a partir de paja de arroz pretratada con hidróxido sódico.

Hidrólisis enzimática	Carga de sólidos (% p/v)	8.0
	Carga enzimática (FPU/g)	15.0
	Tampón	Acetato 50 mM
Fermentación ABE	pH inicial	5.8
	Sulfato de hierro (g/L)	0.02
	Extracto de levadura (g/L)	4.00

A partir de los resultados obtenidos en el FFD preliminar se determinaron, mediante un CCD, los valores de la concentración de hidróxido sódico y del tiempo de reacción que maximizaban la relación butanol-biomasa a las 72 horas, mientras que la temperatura de reacción y la carga de sólidos se fijaron, respectivamente, en 134 °C y 5% (p/v). La Tabla 5.12 detalla los valores codificados y reales de las dos variables independientes estudiadas.

Tabla 5.12. CCD de 2 variables para el pretratamiento alcalino de la paja de arroz. α = 1.41.

	Variables independientes	Valores codificados y reales				
		Nivel -α	Nivel -1	Punto central (0)	Nivel +1	Nivel +α
Z_1	Concentración NaOH (% p/v)	0.00	0.15	0.50	0.86	1.01
Z_2	Tiempo (min)	20	26	40	54	60

Las condiciones de operación de la hidrólisis enzimática y de la fermentación fueron las mismas que las del FFD preliminar (Tabla 5.11). Con la finalidad de comprobar la capacidad de predicción del modelo de regresión de la relación butanol-biomasa a las 72 horas en función de las variables de estudio se llevó a cabo un experimento de validación por triplicado.

5.3.3. Desarrollo del proceso SSFR en modo de alimentación semicontinuo

Tras demostrar que el proceso SSF era una configuración eficiente en la producción de biobutanol a partir de paja de arroz y mejorar la etapa de pretratamiento, se desarrolló una configuración avanzada que combinaba el proceso SSF y la recuperación *in situ* de producto (SSFR) y que operaba en modo de alimentación semicontinuo. En este último estudio de la tesis doctoral se utilizó paja de arroz pretratada según las condiciones establecidas en el segundo estudio: una temperatura de 134 °C, un tiempo de 20 minutos, una concentración de hidróxido sódico del 0.75% (p/v) y una carga de sólidos del 5% (p/v).

Como paso previo se llevó a cabo la optimización del proceso SSF en modo discontinuo. Mediante un CCD se obtuvo el modelo de regresión de segundo orden de la producción de biobutanol en función de las variables de estudio: carga de sólidos y carga enzimática. La Tabla 5.13 detalla los valores codificados y reales de las dos variables independientes estudiadas.

Tabla 5.13. CCD de 2 variables para el proceso SSF a partir de paja de arroz pretratada con hidróxido sódico. α = 1.41.

	Variables independientes	Valores codificados y reales				
		Nivel -α	Nivel -1	Punto central (0)	Nivel +1	Nivel +α
X_1	Carga de sólidos (% p/v)	3.8	5.0	8.0	11.0	12.2
X_2	Carga enzimática (FPU/g)	3.7	7.0	15.0	23.0	26.3

La concentración de butanol producido a las 24 horas se seleccionó como variable respuesta, ya que se buscaba mantener una alta productividad para mejorar la recuperación *in situ* de producto. En tales ensayos, se obtuvo a las 24 horas más del 80% del butanol con respecto a la producción a las 72 horas. La Tabla 5.14 resume las condiciones de operación fijadas. En referencia al pH inicial, se mantuvo el valor original de las muestras (7.4) debido a la incapacidad de control de esta variable al trabajar con altas cargas de sólidos.

Tabla 5.14. Condiciones de operación fijadas en el proceso SSF a partir de paja de arroz pretratada con hidróxido sódico.

Hidrólisis enzimática + fermentación ABE	pH inicial	7.4
	Sulfato de hierro (g/L)	0.02
	Extracto de levadura (g/L)	4.00

El rendimiento de la hidrólisis enzimática se determinó mediante un experimento control por triplicado en el que se seleccionó una carga de sólidos del 8% (p/v) y una carga enzimática de 15 FPU/g. La capacidad de predicción del modelo de regresión de la producción de butanol a las 24 horas en función de las variables de estudio se comprobó mediante un experimento de validación por triplicado. Tras optimizar el proceso SSF en modo discontinuo, se llevó a cabo un ensayo con mayor volumen de trabajo (500 mL) en un STR de 2 litros. En base a los resultados del CCD, en este ensayo se seleccionó una carga de sólidos del 9.2% (p/v) y una carga enzimática de 19.9 FPU/g.

Finalmente, se evaluó el proceso SSFR en modo semicontinuo en la producción de biobutanol. Además, con el fin de mejorar la viabilidad económica del proceso, se establecieron tres experimentos en los que se estudió el efecto que tiene una reducción de la carga enzimática y/o de los componentes del medio (compuestos tampón, extracto de levadura y minerales) sobre la producción acumulativa de biobutanol. Estos tres experimentos, iniciados de igual forma que el ensayo SSF en modo discontinuo con 500 mL de volumen de trabajo, se diferenciaron en la adición de mezcla Cellic® CTec2 y de componentes del medio

junto con la alimentación de biomasa a las 20 y 30 horas, tal y como resume la Tabla 5.15. En el experimento 1, la mezcla Cellic® CTec2 se añadió para mantener una carga enzimática constante de 19.9 FPU/g a lo largo de todo el proceso, mientras que en los experimentos 2 y 3 únicamente se añadió al inicio para estudiar el efecto de la reducción de la carga enzimática. En los experimentos 1 y 2, se fijó a lo largo de todo el proceso la misma relación entre la concentración de los componentes del medio y la carga de sólidos que la establecida en el proceso en modo discontinuo, mientras que en el experimento 3 el medio sólo se suplementó al inicio para estudiar el efecto de la reducción de dichos componentes.

Tabla 5.15. Plan experimental en la evaluación del proceso SSFR en modo semicontinuo a partir de paja de arroz pretratada con hidróxido sódico.

Experimento	Adiciones		
	Sólido (% p/v)	Enzima (FPU/g)[a]	Suplementación del medio
1	9.2 + 4.6 + 4.6	10.0 + 10.0 + 10.0	Si + Si + Si
2	9.2 + 4.6 + 4.6	10.0 + 0.0 + 0.0	Si + Si + Si
3	9.2 + 4.6 + 4.6	10.0 + 0.0 + 0.0	Si + No + No

[a] Respecto al sólido añadido.

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6. COMPARISON OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION AND SEPARATE HYDROLYSIS AND FERMENTATION PROCESSES FOR BUTANOL PRODUCTION FROM RICE STRAW

COMPARISON OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION AND SEPARATE HYDROLYSIS AND FERMENTATION PROCESSES FOR BUTANOL PRODUCTION FROM RICE STRAW

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Abstract

Rice straw (RS) is one of the lignocellulosic wastes with the highest global production. The main objective of this study was to maximise the butanol production by *Clostridium beijerinckii* DSM 6422 from RS pretreated by microwave-assisted hydrothermolysis. Two different fermentation strategies were compared: separate hydrolysis and fermentation (SHF, two-step process) and simultaneous saccharification and fermentation (SSF, one-step process). In parallel, the variables that significantly affected the butanol production were screened by using fractional factorial designs. Butanol concentration and productivity at 48 h were, respectively, 8% and 173% higher in SSF than in SHF. A one-step process was more efficient than a two-step process, especially considering the time savings derived from much higher productivity. From these results, SSF was further optimised by response surface methodology with central composite design over the key factors on the butanol production at 48 h: initial pH, enzyme loading and yeast extract concentration. The optimum point yielded a butanol productivity of 0.114 g L^{-1} h^{-1}, with a butanol-biomass ratio of 51 g kg^{-1} of raw RS (ABE-biomass ratio of 77.0 g kg^{-1} of raw RS). The parameter with the greatest effect was enzyme loading, with an optimal value of 13.5 FPU g-dw^{-1}. This study showed that microwave-processed RS has great potential as a substrate for the butanol production from ABE fermentation when combining process stages by SSF.

Highlights

- Simultaneous Saccharification/Fermentation: greater efficiency on biofuel production
- One-step process increased substantially productivity reducing the production cost

- Significant factors in SSF were enzyme loading (with greatest effect) and yeast extract
- Citrate showed an inhibitory effect on *Clostridium beijerinckii*
- Microwave-processed rice straw has great potential to produce butanol

Keywords

Butanol; lignocellulosic waste; microwave thermohydrolysis, rice straw, simultaneous saccharification/fermentation

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6.1. Introduction

The expected increase of the world population by more than 30% in the next 40 years, the depletion of resources, external energy dependence and climate change are altering the way in which biological resources in Europe are managed. In this context, interest in biomass as a source of carbon and energy has increased (European Union: European Commission, 2012). Lignocellulosic material is the most abundant and economical biomass on the planet (Birgen et al., 2019). Numerous raw materials, such as agricultural residues, forestry wastes, industrial and municipal wastes, and bioenergy crops, are available for the production of biofuels, including biobutanol (Bajpai, 2016). However, a pretreatment is necessary to alter the lignocellulosic structure and to remove and/or alter lignin, generally followed by an enzymatic or acid hydrolysis stage to obtain sugar monomers (Ibrahim et al., 2017).

Biobutanol is mainly produced by *Clostridium acetobutylicum* or *C. beijerinckii* in acetone-butanol-ethanol (ABE) fermentation by a pathway consisting of two metabolic phases: acidogenesis, followed by solventogenesis (Abo et al., 2019). During acidogenesis bacterial growth occurs with the production of acids, hydrogen and carbon dioxide; whereas in the solventogenesis stage the production of solvents and endospore formation occurs (Jones and Woods, 1986). These gram-positive and anaerobic bacteria belong to the only genus capable of producing this solvent as a major metabolite (Jiao et al., 2016). Butanol has some benefits as a fuel in terms of energy density, handling, transport and storage (Qureshi et al., 2008). Despite these advantages, its production by fermentation cannot compete economically with the butanol obtained in the petrochemical industry due to, among other causes, lower development of bioprocesses, long fermentation times, high cost of the substrate,

low yields and high cost of product recovery (García et al., 2011). Strategies developed to enhance cellulosic biobutanol production include strain improvement by genetic engineering, optimisation of the medium formulation and combination of ABE fermentation stages (Ibrahim et al., 2017). To screen and optimise the effect of medium conditions and process parameters on ABE productivity, statistical techniques such as fractional factorial design and response surface methodology (RSM) are often used (Mariano et al., 2010).

The processes derived from the combination of ABE fermentation stages are simultaneous saccharification and fermentation (SSF), consolidated bioprocessing (CBP), separate hydrolysis and fermentation with *in-situ* recovery (SHFR) and simultaneous saccharification and fermentation with *in-situ* recovery (SSFR; Ibrahim et al., 2017). SSF was developed by Gauss et al. (1976) and combines enzymatic hydrolysis and ABE fermentation in one step, increasing the butanol yield and productivity compared to separate hydrolysis and fermentation (SHF). SSF could potentially reduce operational costs and the risk of contamination. In addition, the SSF process minimises glucose inhibition on cellulases and β-glucosidase because bacteria consume sugars as soon as they are released (Ibrahim et al., 2017). For example, Qi et al. (2019) observed that butanol production was higher in SSF (12.64 g L^{-1}) than in SHF (11.25 g L^{-1}) by fermenting ammonium sulfite-pretreated wheat straw with *C. acetobutylicum* ATCC 824, despite decreasing the biomass loading from 10.5 to 9% (w/v). Not only was SSF more efficient in terms of butanol production and time, but enzyme loading was reduced by one-half, thereby conferring an economic advantage. However, Shao and Chen (2015) obtained a shorter fermentation time and a higher butanol concentration by the same bacterial strain from *Amorphophallus* konjac waste in SHF, suggesting that the most appropriate process depends on factors such as the feedstock type and the strain of bacteria used.

One of the most abundant lignocellulosic wastes in the world is rice straw (RS), with an estimated annual production of 731 million tons (Kim and Dale, 2004). Unlike other straws, RS is not generally used as animal feed due to its low digestibility and, apparently, it has a low value for social benefit, so it is burnt openly in the field, causing air pollution (Sarkar and Aikat, 2013). There are numerous reported pretreatments (physical, chemical, physicochemical and biological) to enhance ABE fermentation of RS (Vivek et al., 2019). Despite the low lignin content in RS (Hsu et al., 2010), these methods must face other limiting factors, such as the presence of accumulated silica (Ma et al., 2009) and high cellulose crystallinity (Zhu et al., 2005). Among these pretreatment options, dielectric heating by microwave irradiation is used on lignocellulose as an alternative to convection heating (Amiri

and Karimi, 2018). Indeed, Ma et al. (2009) noticed that microwave pretreatment could improve the enzymatic accessibility of cellulose by partially breaking the lignin-hemicellulose structure and the waxy structure of silicon, increasing solubility. Furthermore, Zhu et al. (2005) determined that, compared to the alkali-alone process, microwave-assisted alkali pretreatment eliminates more hemicellulose and lignin from RS, consequently obtaining a hydrolysate with more glucose and less xylose after enzymatic hydrolysis. One of the limitations for the production of biobutanol is the generation during pretreatment of compounds that inhibit microbial growth, such as acetic acid, 5-hydroximethylfurfural (HMF) and furfural (Vivek et al., 2019). After pretreating RS with dilute acid, Hsu et al. (2010) observed a correlation between the generation of these compounds and pretreatment severity. Indeed, Fonseca et al. (2018) demonstrated that detoxification of rice improved the ethanol productivity from RS hydrolysate with dilute acid. Another alternative to overcome the toxicity derived from chemical pretreatment is the use of non-catalysed methods such as microwave irradiation. This strategy can avoid problems of inhibition by these compounds, saving at the same time the cost derived from chemicals. Although SSF processes have been reported for butanol production by ABE fermentation using other agricultural waste such as wheat straw (Qureshi et al., 2008; Qi et al., 2019; Wang et al., 2013), corn stover (Qureshi et al., 2014) or corncob (Boonsombuti et al., 2016), among others, there is no literature data on the effect of using SSF to produce butanol from RS.

The scope of this work is to evaluate the SSF process for butanol production by *C. beijerinckii* DSM 6422 from RS previously treated by microwave-assisted hydrothermolysis. SSF configuration was compared with SHF in terms of butanol productivity by evaluating the effect of the following parameters: type of buffer (citrate or acetate) and enzyme loading for enzymatic hydrolysis; and initial pH, yeast extract concentration and iron concentration in the fermentation broth in two sets of fractional factorial design experiments. In a later stage, SSF was further optimised using RSM with central composite design (CCD) over variables with statistically significant effects.

6.2. Materials and methods

6.2.1. Materials

RS was obtained from local farmers of L'Albufera located near Valencia (Spain). The biomass was dried for 24 h at room temperature, cut into fragments of ~2 cm and milled. Particle size between 100 and 500 μm was selected by ISO-3310.1

sieve (CISA, Spain), afterwards it was dried in an oven at 45 °C until the residual moisture content was less than 5% (w/w), and it was then stored for further use. The comercial enzyme blend Cellic® CTec2 (Novozyme, Denmark) was employed for hydrolysis of the pretreated RS. The cellulase activity of the enzyme was measured according to the method of the National Renewable Energy Laboratory (NREL; Adney and Baker, 1996), resulting in a value of 119 filter paper units (FPU) mL^{-1}.

6.2.2. RS pretreatment

Microwave-assisted hydrothermal hydrolysis was performed in an ETHOS One microwave digestion system (Milestone, Italy). The microwave had a maximum power of 1500 W and was controlled via a microprocessor with a capacity of 10 TFM vessels (an internal temperature sensor was installed in a reference vessel). The RS was pretreated at 10% (w/v) using 3 g of dry biomass in 30 mL of deionized water. The microwave was heated using the following ramp of temperature: an initial increase to 100 °C at a rate of 15 °C min^{-1}, which was then increased at 6 °C min^{-1} until 160 °C and then to 4 °C min^{-1} until 200 °C, holding at 15 min (García-Puchol et al., 2019). Once the heating was finished, the vessels were cooled at room temperature. The slurry was centrifuged at 10000 rpm for 5 min (centrifuge 5804, Eppendorf, Germany), and the solid phase was washed with deionized water and pH was adjusted to 6.5. Finally, the pretreated RS was dried at 45 °C.

6.2.3. Microorganism and inoculum preparation

The bacterial strain *Clostridium beijerinckii* DSM 6422 (NRRL B-592) was supplied by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The strain was stored at −80 °C in a Reinforced Clostridial Medium (RCM) with 20% (v/v) glycerol. Before fermentation, the cells were grown in 50-mL serum bottles containing 40 mL of modified RCM (19 g L^{-1} RCM supplemented with 10 g L^{-1} glucose) under anaerobic conditions by sparging pure nitrogen in the medium. The inoculum was statically incubated at 37 °C for 24 h. The media used in the cryopreservation and the inoculum preparation were sterilized in an autoclave for 21 min at 121 °C.

6.2.4. ABE fermentation

6.2.4.1. ABE fermentation by SHF

Pretreated RS was hydrolysed prior to fermentation in a separate vessel using the commercial enzyme blend Cellic® CTec2. Enzymatic hydrolysis was carried out

in a 100-mL conical flask (with 50 mL of working volume) in a SI500 orbital shaker (Stuart, UK). The hydrolysis process was performed at 50 °C and 150 rpm for 72 h with a biomass loading of 10% (w/v) and an enzyme dosing of 4.1 FPU g-dw^{-1}. The buffer employed was citrate (50 mM) or acetate (50 mM), whose effects on ABE fermentation were assessed using the fractional factorial design of the experiment described in Section 6.2.6. The initial pH was adjusted to 5.0 by NaOH and HCl. After enzymatic hydrolysis, the samples were centrifuged (6 min, 4000 rpm), filtered by 1.2 µm and stored at 4 °C for a maximum of 12 h before fermentation. A volume of 34.6 mL of the enzymatic hydrolysate was fermented in 50-mL serum bottles with a working volume of 40 mL. The concentration of the buffer and the minerals was based on a modified P2 medium introduced by Monot et al. (1982): 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} NH_4OAc, 0.09 g L^{-1} $MgSO_4.7H_2O$ and 0.001 g L^{-1} $MnSO_4$.H2O. The resazurin concentration was set to 1 mg L^{-1}. $FeSO_4.7H_2O$ and yeast extract were added in concentrations of 0.01 or 0.02 g L^{-1} and 2 or 4 g L^{-1} respectively, and the initial fermentation pH was adjusted to 6.4 or 7.4, according to the fractional factorial design of the experiment. Beforehand, the sealed bottles were autoclaved for 10 min at 121 °C; the oxygen was displaced by sparging pure nitrogen. The inoculation was carried out with 2 mL (5% v/v) of actively growing cells, and the serum bottles were incubated at 37 °C and 150 rpm for 72 h.

6.2.4.2. ABE fermentation by SSF

In this configuration, the pretreated RS was simultaneously hydrolysed and fermented in a 50-mL serum bottle (working volume of 40 mL) with a biomass loading of 9% (w/v). The medium for conducting the SSF experiments was the same as that for the SHF experiments, except that no hydrolysis buffer (50 mM citrate or acetate) was added as the fermentation media contained 28.5 mM of acetate. The effect of the same media parameters (iron and yeast extract concentrations) as in SHF was assessed by the fractional factorial design of the experiment. In this case, the initial reaction pH was set to 5.2 or 6.2 as representatives of optimum values for saccharification or fermentation respectively. The oxygen was displaced by sparging pure nitrogen before autoclaving for 10 min at 121 °C. Afterwards, the enzyme was added along with the inoculum. A loading of 4.1 or 12.4 FPU g-dw^{-1} of Cellic® CTec2 was used in the fractional factorial design experiments in order to assess its influence in SSF. The inoculation was carried out with 2 mL (5% v/v) of actively growing cells. The SSF bottles were incubated at 37 °C and 150 rpm for 120 h. Additionally, two independent replicates of a control experiment (without inoculation) were carried out with the maximum enzyme loading (12.4 FPU g-dw^{-1}) at the minimum pH (5.2) in order to evaluate the maximum release of

monosaccharides from the pretreated RS. From results obtained as described herein, CCD was used for further optimisation of the SSF results.

6.2.5. Analytical methods

The structural carbohydrates, lignin and the moisture content of the RS were determined according to the National Renewable Energy Laboratory (NREL) procedures (Sluiter et al., 2008). The characterisation of the fermentation was carried out by the analysis of pH, cell growth, production of acids and solvents, and sugar uptake from 1-mL samples collected at appropriate times. The pH was measured by a Minitrode electrode (Hamilton, USA). Cell density (g-dw L^{-1}) was calculated from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany). The correlation between OD_{600} and cell density was determined as follows: g-dw L^{-1} = 0.2153·OD_{600} + 0.0689 (n = 10, R^2 = 0.9907). Samples were centrifuged at 10000 rpm for 5 min, and the supernatant was filtered by 0.22 µm before chromatographic analysis. Acids (acetic acid and butyric acid) and solvents (butanol, acetone and ethanol) were analysed in a gas chromatograph (TRACE GC Ultra, Thermo Scientific, USA) equipped with a Teknokroma TRB-FFAP capillary column (30 m × 0.25 mm × 0.25 µm), with helium as carrier gas at a flow rate of 1 mL min^{-1}. One microliter of acidified samples was injected at 250 °C (10:1 split ratio), and the compounds were detected in a flame ionization detector at 250 °C. The oven temperature was held at 50 °C for 4 min, increased at 30 °C min^{-1} until 80 °C (hold time 3 min), and increased at 20 °C min^{-1} until 210 °C (hold time 5 min). Sugars (glucose, xylose and arabinose) were analysed by an ion chromatograph (883 Basic IC plus, Metrohm, Switzerland) equipped with an amperometric detector and a Metrosep Carb 2 anion exchanger column (150 mm × 4 mm × 5 µm). The mobile phase (20 mM NaOH) was set at a flow rate of 0.5 mL min^{-1}. Data are the mean of, at least, two technical replicates.

For the evaluation of the process performance, the following parameters were used:

$$\text{Butanol (or ABE)} - \text{biomass ratio}(\text{g kg}^{-1}) = \frac{\text{Butanol (or ABE) produced (g)}/\text{V}_{\text{hydrolisate fermented}}\ \text{(L)}}{[\text{Biomass loading (kg L}^{-1})/\text{Solid recovery (\%)}] \times 100} \quad (1)$$

$$\text{Butanol (or ABE) productivity (g L}^{-1}\text{h}^{-1}) = \frac{\text{Butanol (or ABE) concentration (g L}^{-1})}{\text{Total reaction time (h)}} \quad (2)$$

$$\text{Butanol (or ABE) yield (g g}^{-1}) = \frac{\text{Butanol (or ABE) concentration (g L}^{-1})}{\text{Sugar consumed (g L}^{-1})} \quad (3)$$

To compare the reaction time of the SHF process with that of SSF, the total reaction time of hydrolysis plus fermentation was assessed. Solid recovery refers to biomass recovered after pretreatment expressed as a percentage.

6.2.6. Design of experiments and statistical analysis

In this work, SSF was first assessed and compared with SHF, by fractional factorial designs. As the SSF process performed better than the SHF process, the significant variables for SSF were further optimised by RSM using CCD. The response variable in all cases was the concentration of butanol produced at 48 h. The commercial software MINITAB® v.18.1 (LEAD Technologies, Inc.) was used for the design of experiments, regression analysis and analysis of variance (ANOVA) at a confidence level of 95% (p-value less than 0.05).

6.2.6.1. Fractional factorial design and data analysis

A 2^{4-1} fractional factorial design (resolution IV, 8 experiment runs) was used to identify the significant factors affecting butanol production at 48 h both in SHF and SSF processes. The effects of three variables (low level and high level) - yeast extract concentration (2 and 4 g L^{-1}), $FeSO_4.7H_2O$ concentration (0.01 and 0.02 g L^{-1}) and initial fermentation pH (6.4 and 7.4 for SHF, and 5.2 and 6.2 for SSF, respectively) - were evaluated in both processes. For SHF, the fourth variable was the buffer employed for enzymatic hydrolysis (50 mM acetate and 50 mM citrate), whereas for SSF it was the enzyme loading (4.1 and 12.4 FPU g-dw^{-1}).

6.2.6.2. Central composite design and data analysis

After identification of significant factors, an RSM with CCD was used in the SSF process to determine the optimal combination of enzyme loading, initial pH and yeast extract concentration for maximising butanol production. The established range for each factor was as follows: enzyme loading (from 5.4 to 19.1 FPU g-dw^{-1}), initial pH (from 5.6 to 7.2) and yeast extract concentration (from 0.5 to 5.5 g L^{-1}). Table 6.1 summarises the coded and real values of the three variables used in CCD, which comprised a total of 20 experimental runs with 6 central point replications. Finally, a validation step was carried out by three replicates using the optimised conditions for butanol production.

Table 6.1. 5-Level CCD of 3 variables for the SSF process. α = 1.68.

	Independent variables	Coded and real values				
		Level -α	Level -1	Central point (0)	Level +1	Level +α
Z_1	Enzyme loading (FPU g-dw^{-1})	5.4	8.2	12.2	16.3	19.1
Z_2	Initial pH	5.6	5.9	6.4	6.9	7.2
Z_3	Yeast extract (g L^{-1})	0.5	1.5	3.0	4.5	5.5

6.3. Results and discussion

6.3.1. Pretreatment of RS

Microwave-assisted hydrothermolysis was selected as RS pretreatment since it presents short reaction times, uniform and rapid heating of biomass, lower generation of inhibitory compounds, higher removal of acetyl groups in hemicellulose, and lower costs in comparison with acid or alkaline pretreatments (López-Linares et al., 2019). The chemical compositions of the raw and pretreated RS are presented in Figure 6.1.

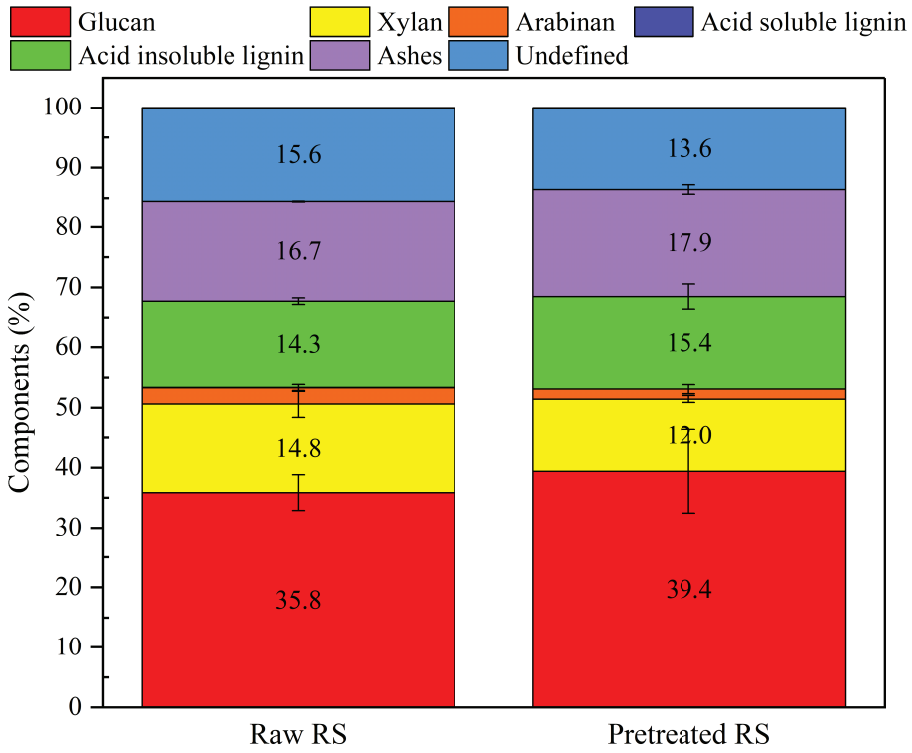

Figure 6.1. Chemical composition of raw and pretreated rice straw.

The untreated dried material consisted of 35.8 ± 2.1% glucan, 14.8 ± 1.6% xylan, 2.7 ± 0.4% arabinan, 0.1 ± 0.0% acid soluble lignin, 14.3 ± 0.4% acid insoluble lignin and 16.7 ± 0.1% ash. This composition is in the typical value range found for

RS of different sources (Singh et al., 2014; Zhao et al., 2018). Recently, Passoth and Sandgren (2019) reported that the typical values for the three major polymers ranged from 29.2 to 34.7% for cellulose, 12.0 to 29.3% for hemicellulose, and 17.0 to 19.0% for lignin, being silica the major ash component.

The pretreatment resulted in a solid recovery of 80.5% of the raw RS with different degrees of degradation among carbohydrate fractions. For example, the glucan percentage increased from 35.8 to 39.4%, although the total percentage of carbohydrates remained almost stable at ~53% due to the loss of hemicellulose. During pretreatment only 11.5% of glucan was lost, but greater degradation of arabinan and xylan was observed, with losses of 50.6% and 34.5% respectively. These results indicated that some hemicellulose was removed in the microwave pretreatment of RS; phenomena also observed by Zhu et al. (2005) at lower irradiation powers. Higher values of polysaccharides were recovered from raw RS (80.1%) when compared to previous studies on this type of biomass. For example, Amiri et al. (2014) obtained a recovery of 76.2% after organosolv pretreatment with a 75% (v/v) ethanol and 1% (w/w) sulfuric acid solution at 150 °C during 30 min, and Moradi et al. (2013) found a value of 51.9% after 3 h of alkaline pretreatment at 0 °C with a 12% (w/v) NaOH solution. The microwave irradiation at the tested conditions resulted in a 13.3% delignification and removed 13.7% of ashes (silica content), thus improving the RS digestibility. However, the delignification degree obtained in this study could impact the saccharification of the waste, since lignin binds non-productively to cellulase due to its hydrophobic nature (Lan et al., 2013). On the other hand, the remaining silica in the pretreated RS could also act as a physical barrier, protecting for enzymatic hydrolysis (Řezanka and Sigler, 2008).

6.3.2. Comparison of ABE fermentation by SHF and SSF: screening of key factors

The 2^{4-1} fractional factorial design was conducted for the SHF and SSF processes to evaluate the influence of the selected parameters on butanol production. The experimental design and the response results for both processes are shown in Table 6.2. The analysis of variance (ANOVA) of the outcomes, with the estimated coefficients and significant levels for the regression model and the evaluated variables for the SHF and SSF processes are shown in supplementary material. In both cases, after 72 h of fermentation the concentrations of butanol and ABE increased less than 1% from 48 h. Therefore, butanol production at 48 h was considered as the response variable. The fast rate of solvent production demonstrated the successful balance between the acidogenic and solventogenic metabolic phases of *Clostridium beijerinckii* DSM 6422 using both the appropriate

operational conditions and the adequate biomass pretreatment. Models for SHF and SSF were statistically significant, with p-values lower than 0.05. In addition, the values of the coefficient of determination (R^2: 0.9996 and 0.9997 for SHF and SSF, respectively) and the adjusted coefficient of determination (Adj. R^2: 0.9975 and 0.9976 for SHF and SSF, respectively) were close to 1.0, indicating the goodness of fit of the models.

Table 6.2. 2^{4-1} fractional factorial design and experimental results for the SHF and for the SSF processes.

SHF Process					
	Real values				Response
Run	Yeast extract (g L^{-1})	$FeSO_4 \cdot 7H_2O$ (g L^{-1})	Initial pH	Saccharification buffer	Butanol 48 h (g L^{-1})
1	2	0.01	6.4	50 mM acetate	4.68
2	2	0.02	6.4	50 mM citrate	3.13
3	2	0.02	7.4	50 mM acetate	4.78
4	2	0.01	7.4	50 mM citrate	2.72
5	4	0.02	6.4	50 mM acetate	4.84
6	4	0.01	6.4	50 mM citrate	3.16
7	4	0.01	7.4	50 mM acetate	4.85
8	4	0.02	7.4	50 mM citrate	3.05
SSF Process					
	Real values				Response
Run	Yeast extract (g L^{-1})	$FeSO_4 \cdot 7H_2O$ (g L^{-1})	Initial pH	Enzyme loading (FPU g-dw^{-1})	Butanol 48 h (g L^{-1})
1	2	0.01	5.2	4.1	1.55
2	2	0.02	5.2	12.4	1.53
3	2	0.02	6.2	4.1	2.80
4	2	0.01	6.2	12.4	4.98
5	4	0.02	5.2	4.1	1.00
6	4	0.01	5.2	12.4	0.58
7	4	0.01	6.2	4.1	1.28
8	4	0.02	6.2	12.4	5.24

From the two-step process (SHF) results, among the four variables screened, only the type of buffer used during enzymatic hydrolysis was found to be significant (p-value of 0.0120). The linear coefficient of the buffer factor (low-high level: acetate-citrate) was lower than zero, indicating that the use of citrate buffer during the saccharification step negatively affected butanol production. Citrate buffer at 50 mM is widely used to maintain a pH around 5.0 during enzymatic hydrolysis (Amiri et al., 2014; Gottumukkala et al., 2013; Moradi et al., 2013). Furthermore, Xue et al. (2017) showed that 60 mM citrate buffer was optimum for ABE fermentation of Jerusalem artichoke stalk with *C. beijerinckii* CC101, lower and

higher values decreased solvent production. Contrarily, Liu et al. (2015) observed that *C. beijerinckii* NCIMB 8052 did not grow with 50 mM citrate; whereas when acetate was used as a buffer ABE fermentation was not inhibited. In our study, butanol concentrations ranged from 2.72 to 3.16 g L^{-1} by using citrate buffer, while a minimum of 4.68 g L^{-1} was obtained in the experiments with acetate as hydrolysis buffer. Our results corroborated that the use of citrate buffer provokes a negative effect on ABE fermentation of pretreated RS by *C. beijerinckii* DSM 6422. The yeast extract concentration did not show a significant effect in the tested range of 2 to 4 g L^{-1}. Contrarily, a significant impact on the production of butanol by *C. acetobutylicum* MTCC 481 from RS hydrolysate was previously observed, with an optimal concentration of 3 g L^{-1} (Ranjan et al., 2013). Thus, this demonstrates the importance of the preliminary screening of the media composition for each specific lignocellulosic waste and bacterial strain. The non-significant effect of iron on butanol production from RS hydrolysate indicates that the quantity containing the raw material along with the amount from the minimum yeast extract concentration supplied to the fermentation broth is sufficient. Gottumukkala et al. (2013) determined that the improvement in solvent production by *C. sporogenes* BE01 after removing mineral supplementation from RS hydrolysate could be due to the presence of these minerals in the raw material. Ranjan et al. (2013) also found that iron concentration had no impact on ABE fermentation of RS with *C. acetobutylicum* MTCC 481 supplemented with 3 g L^{-1} of yeast extract. Furthermore, the initial fermentation pH was incorporated into the experimental design since it would affect the biochemical and biophysical characteristics of the solventogenic *Clostridium* spp. (Millat and Winzer, 2017). Fermentation pH, together with the rate of acid production, is one potential key factor in the concentration of undissociated acids that can inhibit a correct shift towards solventogenesis (Maddox et al., 2000). In contrast, fermentation pH was found to be non-significant, likely because the sugar concentration released from enzymatic hydrolysis was not sufficiently high enough to unbalance the rate of acid production.

In the case of the one-step process (SSF), two variables were found to be statistically significant. The initial pH had a great effect on butanol production (p-value of 0.0164). Furthermore, the enzyme loading was also significant (p-value of 0.0277). Based on the coded coefficients of the linear effects, the order of importance was as follows: initial pH (1.2041) > enzyme loading (0.7130). These results show that, for SSF, it is better not to use a value near to the optimum for the saccharification of cellulosic materials as the initial pH. Although enzymatic hydrolysis will proceed slowly, solventogenic shift would be favoured. Even though no interaction between initial fermentation pH and enzyme loading had a significant

effect, the need to use a pH above the optimum for enzymatic hydrolysis could explain the higher enzyme loading required in SSF (12.4 FPU g-dw^{-1}) to achieve butanol concentrations above 4 g L^{-1} compared with the SHF process (4.1 FPU g-dw^{-1}). Furthermore, the *in-situ* ABE products in SSF can be linked to the higher enzyme requirements, as they have been shown as inhibitors of the cellulolytic and hemicellulolytic enzyme activity (Qi et al., 2014). Contrarily, the fermentation temperature selected (37 °C) has been reported as a more suitable temperature in comparison to 50 °C for better cellulase and xylanase activities in the presence of ABE products (Qi et al., 2014).

Both configurations were compared in terms of process efficiency. Table 6.3 summarises the experimental data obtained in the runs with the highest butanol production at 48 h for each configuration (run 7-SHF, run 8-SSF). The values of released sugars (g L^{-1}), butanol and ABE production (g L^{-1}), butanol and ABE yield (g g^{-1}), butanol and ABE-biomass ratio (g kg RS^{-1}), and butanol and ABE productivity (g L^{-1} h^{-1}) are included. After 72 h of enzymatic hydrolysis (SHF), the concentrations obtained of glucose, xylose and arabinose were 17.68, 6.10 and 0.39 g L^{-1} respectively. In order to evaluate the maximum sugars released in the SSF processes, two control saccharification assays without inoculum (initial pH = 5.2, enzyme loading = 12.4 FPU g-dw^{-1}) were carried out, with average glucose, xylose and arabinose concentrations of 18.92, 6.91 and 0.64 g L^{-1} respectively. Regardless of the process used, the concentrations of sugars released by the enzyme blend Cellic® CTec2 from the pretreated RS were very similar (~50% sugar recovery). Thus, corroborating the idea that the need for higher enzyme dosing in SSF than in SHF relies on the enzymatic inhibition by ABE products and/or on the impossibility of performing the saccharification at the optimum pH. The delignification (13.3%) combined with the ash removal (13.7%) achieved after microwave pretreatment limited, to some extent, the sugar recovery from enzymatic hydrolysis. Concerning the SHF configuration, the butanol concentration at 48 h was 4.85 g L^{-1} with an ABE concentration of 7.95 g L^{-1} (butanol:acetone mass ratio of 1.6:1, ethanol was not detected). All the released sugars were consumed at the end of the fermentation, resulting in a butanol (ABE) yield of 0.245 g g^{-1} (0.402 g g^{-1}). The RS exploitation was evaluated with the butanol or ABE-biomass ratio, with observed values of 44.6 g of butanol and 73.1 g of ABE per kg of raw RS. In the SSF process, the butanol concentration at 48 h of reaction time (5.24 g L^{-1}) increased by 8% of that observed in SHF and, in turn, the butanol-biomass ratio rose to 48.2 g kg RS^{-1}; whereas the ABE concentration (butanol:acetone mass ratio of 1.8:1, ethanol was not detected) increased only by 3.7%. The total concentration of sugars in the fermentation broth was less than 1.5 g L^{-1} and the butanol (ABE) yield resulted in 0.217 g g^{-1} (0.341 g

g^{-1}) considering the maximum sugar concentration released in the two control experiments. It should be noted that both glucose and xylose were nearly completely consumed by the microorganisms, thus, maximum utilisation of the sugars released in the saccharification step was reached. Guan et al. (2016) also pointed out that the SHF process showed higher ABE yields than those obtained in the SSF process from fermenting Kraft paper mill sludge by *C. acetobutylicum* ATCC 824.

Table 6.3. Comparison of SHF and SSF processes after 48 h of fermentation.

Method	Released sugars (g L^{-1})[a]		Butanol (ABE) production (g L^{-1})	Butanol (ABE) yield (g g^{-1})	Butanol (ABE)-biomass ratio (g kg RS^{-1})	Butanol (ABE) productivity (g L^{-1} h^{-1})
	Glucose	Xylose				
SHF	17.68	6.10	4.85 (7.95)	0.245 (0.402)	44.6 (73.1)	0.040 (0.066)
SSF	18.92	6.91	5.24 (8.24)	0.217 (0.341)	48.2 (75.8)	0.109 (0.172)

[a] Sugars obtained after 72 h of hydrolysis time. In the case of SSF, sugars released from two abiotic controls

The most remarkable difference between the one-step and two-step processes was found in the overall butanol productivity. A productivity of 0.040 g L^{-1} h^{-1} was achieved in the SHF process, while a value 2.7-fold higher (0.109 g L^{-1} h^{-1}) was reached in the SSF process. This greater productivity is related not so much to the increase (by 8%) in the final butanol concentration but instead to the lower operation time needed to carry out the valorisation process of the RS. The SHF process needed a total of 120 h (72 h of enzymatic hydrolysis followed by 48 h of fermentation), while in the SSF process only 48 h were required to complete the butanol production at the same or even slightly higher levels than in the two-step process. Furthermore, the SSF process showed greater exploitation of the RS with a higher butanol-biomass ratio. Other authors compared the simultaneous process to the conventional SHF in the production of butanol from wheat straw (Qureshi et al., 2008; Qi et al., 2019), showing that SSF was more efficient and time-saving than SHF. Our results corroborated previous findings, revealing the potential of SSF to be less expensive than SHF in butanol production from the hydrolysate of straw. The greater efficiency of the SSF process could imply a reduction in equipment investment (only one vessel is necessary) and operational costs (lower production times, less contamination risk) in the production of butanol from RS.

6.3.3. Optimization of butanol production by SSF

Based on the above results, a RSM with full factorial CCD was performed for the SSF process to maximise butanol production by optimising three factors: enzyme

loading, initial pH and yeast extract concentration. The model was validated by performing an experiment, with 3 replicates, at the optimum conditions.

6.3.3.1. Response surface methodology

The response surface methodology approach consisted of a five-level, three-factor CCD (Table 6.1) and subsequent linear regression analysis to fit the experimental data with a second-order model. Three independent variables were selected for the determination of the main effects and their interactions on butanol production. Enzyme loading (Z_1) and initial pH (Z_2), were found to be significant in the fractional factorial design of the SSF process, whereas yeast extract concentration (Z_3) was included for further study by enlarging its variation range. Based on the previous results, $FeSO_4.7H_2O$ concentration was set to 0.02 g L^{-1}. Table 6.4 shows the CCD experimental matrix with variables in real terms and the observed and predicted values of butanol concentration after 48 h obtained from each condition. A total of 20 experimental runs were carried out, including 6 central point replications to check the experimental variability.

Table 6.4. CCD experimental matrix along with the observed and predicted values of the response for the SSF process.

Run	Real values			Butanol (g L^{-1})	
	Z_1	Z_2	Z_3	Observed	Predicted
1	8.2	5.9	1.5	2.62	2.22
2	16.3	5.9	1.5	2.52	2.96
3	8.2	6.9	1.5	2.79	2.70
4	16.3	6.9	1.5	3.68	3.99
5	8.2	5.9	4.5	4.21	4.00
6	16.3	5.9	4.5	4.62	4.82
7	8.2	6.9	4.5	3.83	3.50
8	16.3	6.9	4.5	4.38	4.88
9	5.4	6.4	3.0	1.06	1.72
10	19.1	6.4	3.0	4.31	3.50
11	12.2	5.6	3.0	4.14	4.17
12	12.2	7.2	3.0	4.80	4.62
13	12.2	6.4	0.5	3.11	3.01
14	12.2	6.4	5.5	5.31	5.25
15	12.2	6.4	3.0	n.a.[a]	4.96
16	12.2	6.4	3.0	4.58	4.96
17	12.2	6.4	3.0	5.19	4.96
18	12.2	6.4	3.0	5.10	4.96
19	12.2	6.4	3.0	4.81	4.96
20	12.2	6.4	3.0	5.10	4.96

[a] n.a.: non available

The experimental results showed that the one-step process succeeded in producing butanol after 48 h within the ranges of the independent variables, achieving butanol concentrations from 1.06 to 5.31 g L^{-1}. Data of run 15 was not included due to oxygen contamination detected by a resazurin indicator. The greater butanol production (5.31 g L^{-1}) was obtained in run 14 with an ABE concentration of 8.48 g L^{-1} and a butanol:acetone mass ratio of 1.7:1 (ethanol was not detected). Furthermore, a butanol productivity of 0.111 g L^{-1} h^{-1} was reached with a butanol yield of 0.298 g g of consumed $sugar^{-1}$ and a butanol-biomass ratio of 48.8 g per kg of raw RS (ABE-biomass ratio of 78.0 g per kg of raw RS). The second-order model obtained for the concentration of butanol (g L^{-1}) in terms of actual factors was as follows:

$$\begin{aligned}\text{Butanol concentration} = {} & -41.9 + 1.018Z_1 + 10.69Z_2 + 3.26Z_3 - 0.0641Z_1^2 \\ & - 0.804Z_2^2 - 0.1304Z_3^2 + 0.0783Z_1Z_2 \\ & + 0.0039Z_1Z_3 - 0.324Z_2Z_3 \end{aligned} \quad (4)$$

The analysis of variance (ANOVA) and coded regression coefficients of the second-order model for butanol production are presented in Table 6.5. The model was highly significant at the 95% significance level, with a p-value of 0.0015, whereas the lack-of-fit was not significant (p-value of 0.0500). The low standard deviation (SD) value of 0.5037 g L^{-1}, measured in the units of the response variable indicates that the data values are not far from the fitted values. The coefficient of determination (R^2) value was 0.9004, showing a good correlation between the experimental results and the predicted values, in which only 9.96% of the total variations were not explained by the model. The goodness of the predictions was also confirmed by the adjusted coefficient of determination (Adj. R^2: 0.8008), suggesting that this model could properly predict the effect of enzyme loading, initial pH and yeast extract on butanol production after 48 h from RS by SSF. As can be seen from the ANOVA of the model, only the linear coefficients of enzyme loading (Z_1) and yeast extract concentration (Z_3) were found to be significant (p-value of 0.0037 and 0.0009, respectively), whereas initial pH was not significant (p-value of 0.3531). Unlike in other SSF processes (Razali et al., 2018), the variation of the initial pH in the range studied was not crucial on the response, because the effect of this factor depends on the strain, raw material and type of pretreatment (Xue et al., 2017). The coded coefficients of the significant linear effects showed the degree of importance of the factors on the response: yeast extract (0.6671) > enzyme (0.5290). The p-value of the quadratic effect of enzyme (Z_1Z_1) was 0.0002, indicating that this variable had the greatest effect on butanol production. The rest of quadratic and two-way interaction effects were found to be not significant.

Table 6.5. ANOVA of the second-order model for butanol production by SSF process.

Source	Degrees of freedom	Sum of squares	Mean square	F value	p-value Prob > F	Coeffi-cient[a]
Model	9	20.6456	2.2940	9.04	0.0015	
Linear	3	10.1421	3.3807	13.33	0.0012	
Z_1: enzyme loading	1	3.8221	3.8221	15.07	0.0037	0.5290
Z_2: initial pH	1	0.2432	0.2432	0.96	0.3531	0.1334
Z_3: yeast extract	1	6.0768	6.0768	23.95	0.0009	0.6671
Square	3	9.8683	3.2894	12.97	0.0013	
Z_1Z_1	1	9.4120	9.4120	37.10	0.0002	-0.8304
Z_2Z_2	1	0.5516	0.5516	2.17	0.1744	-0.2010
Z_3Z_3	1	1.1750	1.1750	4.63	0.0598	-0.2934
2-way interactions	3	0.6353	0.2118	0.83	0.5079	
Z_1Z_2	1	0.1591	0.1591	0.63	0.4488	0.1410
Z_1Z_3	1	0.0035	0.0035	0.01	0.9086	0.0210
Z_2Z_3	1	0.4726	0.4726	1.86	0.2054	-0.2431
Error	9	2.2832	0.2537			
Lack-of-fit	5	2.0244	0.4049	6.26	0.0500	
Pure error	4	0.2588	0.0647			
Total	18	22.9288				
Standard Deviation, SD					0.5037	
R^2					0.9004	
Adj. R^2					0.8008	

[a] For coded variables

6.3.3.2. Conjugated effect of enzyme and yeast extract

The response surface plot of the final model equation is shown in Figure 6.2, where the combined effect of enzyme loading and yeast extract on butanol production at a constant initial pH of 6.4 (central point in the CCD) is presented. In this figure, the three-dimensional surface and the two-dimensional contours for the butanol concentration after 48 h are plotted. The surface plot shape shows the great effect of the enzyme loading in comparison with the effect of yeast extract concentration. In addition, the rounded shape of the contour plots reflects, besides ANOVA outcomes, that the interaction effect between both factors was weak. As it can be seen, there is a maximum on the butanol concentration within the range of the variables established in the experimental design. According to the second-order model, the optimal conditions of the significant factors needed to achieve a butanol concentration of 5.43 g L^{-1} were an enzyme loading of 13.5 FPU g-dw^{-1} and a yeast extract concentration of 4.7 g L^{-1}. It should be noted that an enzyme loading higher than 16.3 FPU g-dw^{-1} caused a sudden decrease in the butanol concentration. In the

one-step process, apart from increasing the operational costs, a large enzyme load could be counterproductive by inhibiting bacterial growth, as other authors have already pointed out (Razali et al., 2018). Yeast is essential for ABE fermentation from bacteria such as *C. acetobutylicum* DSM 792, unlike other sources of nitrogen such as NH_4Cl and $NaNO_3$ (Raganati et al., 2015). Bacteria use nitrogen in the formation of nucleic acids, proteins and cell wall components (Rehm and Burkovski, 2011), so the increase in yeast extract concentration is usually related to the improvement of growth, which would lead to an increase in sugar consumption and a greater butanol production (Magalhães et al., 2018). However, Al-Shorgani et al. (2018) observed that an excessive reduction of the C/N ratio inhibits butanol production despite favouring the growth of *C. acetobutylicum* YM1.

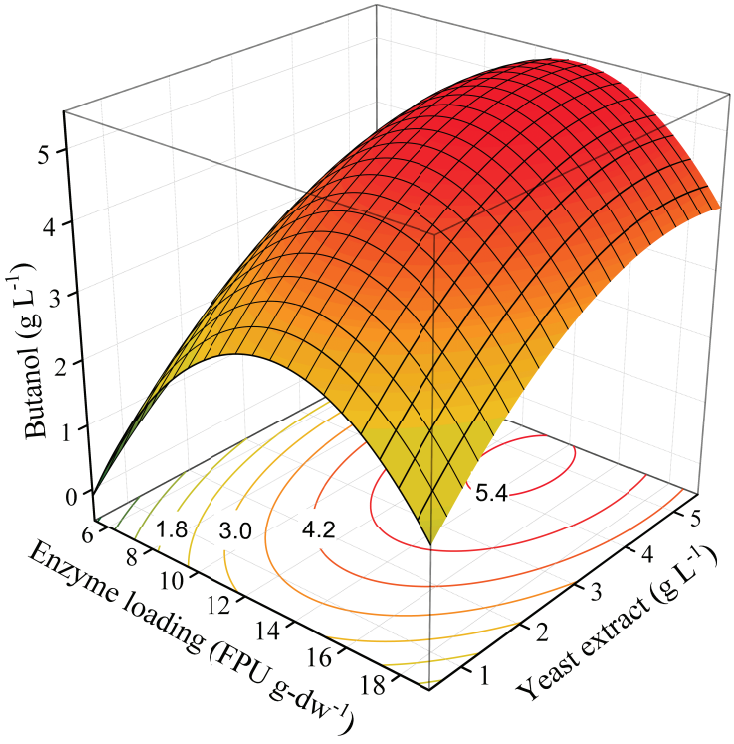

Figure 6.2. The response surface and the corresponding contour plot for butanol production (g L^{-1}) at 48 h in the SSF process: combined effect of enzyme loading (FPU g-dw^{-1}) and yeast extract concentration (g L^{-1}). Initial pH = 6.4.

6.3.3.3. SSF model validation

The validation of the predicted optimal conditions from the CCD results was carried out in three replicates by using an enzyme loading of 13.5 FPU g-dw^{-1}, a yeast extract concentration of 4.7 g L^{-1} and an initial pH of 6.4. The variation with time of the solvent concentration (acetone and butanol; ethanol was not detected), acid concentration (acetic and butyric acid), sugar concentration (glucose, xylose and arabinose) and pH are plotted in Figure 6.3 a. Butanol concentration at 48 h (5.49 ±

0.09 g L^{-1}) only differed by 1.09% from the value estimated from the model (5.43 g L^{-1}), suggesting the goodness of model fit to predict the butanol concentration. Butanol yield and productivity were obtained as 0.306 ± 0.004 g g of consumed $sugar^{-1}$ and 0.114 ± 0.002 g L^{-1} h^{-1} respectively. No increase in butanol concentration was observed after 48 h. Interestingly, 93% of the maximum value was already reached at 24 h, giving a productivity of 0.212 ± 0.004 g L^{-1} h^{-1}. The production of solvents resulted in 8.00 ± 0.10 g L^{-1} of total ABE at 24 h, when the concentration of acetone reaches its highest value (2.92 ± 0.04 g L^{-1}), and 8.40 ± 0.15 g L^{-1} at 48 h. Ethanol was not detected in significant concentrations throughout the study, which is positive for further downstream. Glucose and xylose accumulation were observed during the first 12 h, then decreased rapidly, indicating that enzyme hydrolysis was not the rate-limiting step unlike bacterial metabolism. This reversed after 48 h, when a slight increase in sugars was observed in the fermentation broth. For comparison purposes, the run 7 of the 2^{4-1} fractional factorial design of the SHF processes (highest butanol production for this configuration) was included in Figure 6.3 b. One of the main observable differences is that in order to achieve the maximum butanol production (4.85 g L^{-1}), 72 extra hours are required compared to the one-step process.

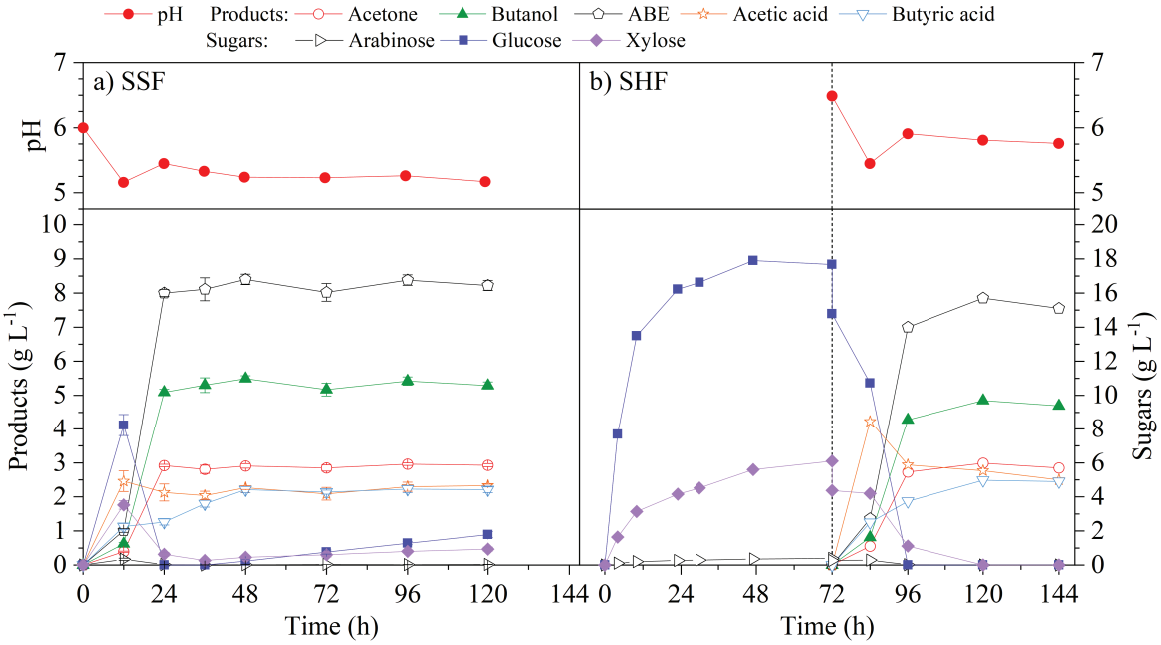

Figure 6.3. Comparison of SSF and SHF processes. (a) SSF: CCD model validation at the predicted optimum conditions. Standard bar errors from three replicates; (b) SHF: best results achieved, single run 7 of the 2^{4-1} fractional factorial design.

A comparison of the results of this study with those derived from the SHF and SSF processes reported in the literature is summarised in Table 6.6. Among the studies presented, different species of the genus *Clostridium* and different

lignocellulosic substrates were used. When comparing butanol and ABE production, the achieved concentrations (5.5 and 8.4 g L^{-1}, respectively) were within the published values (4.0 – 12.6 g L^{-1} for butanol and 7.4 – 19.8 g L^{-1} for ABE), although they were in the lower range due to the low sugar concentration derived from the hydrolizated RS. The yield values of butanol and ABE found in this study (0.31 g g^{-1} and 0.47 g g^{-1}) were much higher than those achieved in the literature (0.16 – 0.20 g g^{-1} and 0.26 – 0.30 g g^{-1}), thus corroborating the notion that solvent production was limited not due to the capacity of the bacterial strain but rather to the limited release of sugars from the lignocellulosic material (22.98 g L^{-1}). This restriction is also indicated by the butanol (51 g kg RS^{-1}) and ABE-biomass ratio (77 g kg RS^{-1}); parameters reflecting the overall conversion from raw RS to solvents that need to be increased for a large-scale production. Besides the high yield, the productivity of butanol (0.11 g L^{-1} h^{-1}) and ABE solvents (0.18 g L^{-1} h^{-1}) was higher than that previously reported for the SSF process, where it takes between 72 and 144 h to reach the maximum concentration of butanol, unlike the 48 h required in our study. Compared with SHF from the literature, the values were even better, as reported productivities do not take into account the required time for the biomass saccharification (48 – 72 h more). This is of great interest, as high productivities are necessary to ensure an adequate butanol removal rate in *in-situ* product removal processes (Moon et al., 2011).

Table 6.6. Comparison of ABE fermentation through SHF and SSF processes from different feedstocks.

Substrate	Pretreatment	Fermentation method	Pretreated biomass loading	Enzyme loading
Brewer´s spent grain	Dilute acid hydrolysis	SHF	10% (w/w)	Celluclast 1.5L (15 FPU g-dw^{-1}), Novozyme 188 (15 IU g-dw^{-1})
Rice straw	Ethanol organosolv	SHF	8% (w/w)	Celluclast 1.5L (25 FPU g-dw^{-1}), Novozyme 188 (40 IU g-dw^{-1})
Paper sludge	None	SSF	5% (w/v)	Cellic CTec2 (15 FPU g $glucan^{-1}$)
Oil palm empty fruit bunch	Alkaline	SSF	5% (w/v)	Acremonium cellulase (15 FPU g-dw^{-1})
Wheat straw	Ammonium sulfite	SSF	9% (w/v)	Cellulase (5 FPU g-dw^{-1}), Xylanase (10 IU g-dw^{-1})
Rice straw	Microwave assisted hydrothermolysis	SSF	9% (w/v)	Cellic CTec2 (12 FPU g $glucan^{-1}$)

Further study is necessary in order to increase the release of sugars from the RS by enhancing the pretreatment method. The use of large concentrations of biomass can lead to problems such as inappropriate energy efficiency in microwave pretreatment (Ma et al., 2009), decrease of mass transfer (Bellido et al., 2015) and decrease of substrate conversion due to enzymatic inhibition (Puri et al., 2013). Therefore, investigations will be focus on improving the delignification and ash removal rather than to increase the biomass loading.

6.4. Conclusions

The serious environmental problems arising from the consumption of fossil fuels are increasing interest in producing biobutanol from lignocellulosic waste as a promising alternative energy source. In this study we demonstrated the feasibility of using hydrolysed rice straw by microwave irradiation as a substrate. By an adequate selection of operational conditions, fermentation time was reduced to 48 h with nearly total consumption not only of glucose, but also of xylose, resulting in high productivity which is a great advantage for scaling-up. Besides, the SSF process was shown to be a favourable configuration with the potential capability to reduce substantially the production cost when compared with a conventional SHF process. From these promising results, further research on pretreatment conditions in order to improve the release of sugar concentrations from saccharification are of great interest to increase the butanol-biomass ratio prior to scale-up.

Table 6.6. cont. Comparison of ABE fermentation through SHF and SSF processes from different feedstocks.

Microorganism	Butanol (ABE) production (g L^{-1})	Butanol (ABE) yield (g g^{-1})	Butanol (ABE)-biomass ratio (g kg RS^{-1})	Butanol (ABE) productivity (g L^{-1} h^{-1})	Reference
C. beijerinckii DSM 6422	6.1 (8.2)	0.20 (0.26)	28 (38)	0.06 (0.08)**	Plaza et al. (2017)
C. acetobutylicum NRRL B-591	7.1 (10.5)	-	70 (103)	0.10 (0.15)**	Amiri et al. (2014)
C. acetobutylicum ATCC 824	8.5 (14.5)	0.18 (0.30)	92 (157)	0.07 (0.12)	Guan et al. (2016)
C. acetobutylicum ATCC 824	4.0 (7.4)	0.16 (0.30)	80 (148)*	0.03 (0.06)	Razali et al. (2018)
C. acetobutylicum ATCC 824	12.6 (19.8)	-	110 (173)	0.09 (0.14)	Qi et al.(2019)
C. beijerinckii DSM 6422	5.5 (8.4)	0.31 (0.47)	51 (77)	0.11 (0.18)	This study

* The butanol and ABE-biomass ratio was calculated considering that the solid recovery was 100%

** The butanol and ABE productivity was calculated without considering the enzymatic hydrolysis time

Appendix A. Supplementary data

Table 6.S1. ANOVA of the 2^{4-1} fractional factorial design for the SHF and for the SSF processes.

Source	Signifi-cant	Sum of squares	Degrees of freedom	Mean square	F value	p-value Prob > F	Coeffi-cient[a]
Statistics of regression for the SHF process							
Model	Yes	6.4374	6	1.0729	475.58	0.0351	
Yeast extract (g L^{-1})	No	0.0432	1	0.0432	19.14	0.1431	0.0735
$FeSO_4 \cdot 7H_2O$ (g L^{-1})	No	0.0186	1	0.0186	8.27	0.2131	0.0483
Initial pH	No	0.0200	1	0.0200	8.85	0.2064	-0.0500
Saccharifi-cation buffer	Yes	6.2990	1	6.2990	2792.12	0.0120	-0.8873
R^2						0.9996	
Adj. R^2						0.9975	
Statistics of regression for the SSF process							
Model	Yes	22.8461	6	3.8077	493.34	0.0344	
Yeast extract (g L^{-1})	No	0.9526	1	0.9526	123.42	0.0571	-0.3451
$FeSO_4 \cdot 7H_2O$ (g L^{-1})	No	0.5923	1	0.5923	76.74	0.0724	0.2721
Initial pH	Yes	11.5984	1	11.5984	1502.72	0.0164	1.2041
Enzyme (FPU g-dw^{-1})	Yes	4.0666	1	4.0666	526.88	0.0277	0.7130
R^2						0.9997	
Adj. R^2						0.9976	

[a] For coded variables

CRediT authorship contribution statement

Alejo Valles: investigation, formal analysis, writing-original draft, visualization. **F. Javier Álvarez-Hornos:** conceptualization, methodology, investigation, writing-review and editing, supervision. **Vicente Martínez-Soria:** conceptualization, methodology, visualization. **Paula Marzal:** conceptualization, methodology, resources. **Carmen Gabaldón:** conceptualization, methodology, investigation, writing-review and editing, supervision, project administration, funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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7. OPTIMIZATION OF ALKALI PRETREATMENT TO ENHANCE RICE STRAW CONVERSION TO BUTANOL

OPTIMIZATION OF ALKALI PRETREATMENT TO ENHANCE RICE STRAW CONVERSION TO BUTANON

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Abstract

The use of rice straw (RS) was enhanced to produce biobutanol as biofuel, for which the NaOH pretreatment was optimized by considering the butanol-biomass ratio that quantify the mass balance efficiency of the three sequential stages of the process: pretreatment, enzymatic hydrolysis and fermentation by *Clostridium beijerinckii*. The optimum point (solid loading of 5% w/v with 0.75% w/v NaOH at 134 °C for 20 min) of the best cost-wise option yielded an enhanced biomass use of 77.6 g kg RS^{-1}. A maximum butanol titer of 10.1 g L^{-1} was reached after 72 h of fermentation with the complete uptake of glucose and nearly complete uptake of xylose. The NaOH concentration was the most influential parameter. The appropriate dosage to maximize fermentable sugars instead of the mass balance efficiency of the three stages underestimated the biomass use by 13%, showing the importance of correctly selecting the variable response during optimization.

Highlights

- Pretreatment rice straw with NaOH was optimized to enhanced biobutanol production
- A novel approach considering the mass balance efficiency of the process was used
- High rice straw exploitation (77.6 g kg^{-1}) and butanol titer (10.1 g L^{-1}) were achieved
- Significant factors were NaOH concentration (with greatest effect) and time

Keywords

Alkaline pretreatment; biofuels; butanol; *Clostridium beijerinckii*; rice straw

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7.1. Introduction

In 2020 the European Commission updated its First Circular Economy Action Plan launched in 2015 (European Union: European Commission, 2020). Among the actions derived from the EU's agenda for sustainable growth, the recast Renewable Energy Directive (EU) 2018/2001 laid down a target for advanced biofuels contributing 3.5% of the total transport sector energy. These biofuels must be produced from feedstocks like lignocellulosic biomass (bagasse, straw, or forestry waste), food cellulosic materials, animal manure or algae, among others. Rice straw (RS), mainly composed of cellulose and hemicellulose, is a promising feedstock with a global production in the range of 370 – 520 million tons per year (Van Hung et al., 2020). Alcohols like methanol, ethanol, propanols, and butanols have been proposed as biofuel candidates. Although butanol has several advantages over shorter alcohols including higher volumetric energy content, it is less corrosive and is more compatible with conventional fuels (Schubert, 2020).

ABE anaerobic fermentation is a sustainable method of producing biobutanol from rice straw. Clostridia species such as *Clostridium acetobutylicum* and *Clostridium beijerinckii* carry out ABE fermentation through biphasic metabolism: the organic acids produced in the acidogenic phase are re-assimilated later in the solventogenic phase to produce acetone, butanol and ethanol (Jones and Woods, 1986). Although there are some shortcoming in batch fermentation (product inhibition, dead time periods required for medium preparation and sterilization) which result in lower butanol productivity, batch configuration has been conventionally selected for industrial ABE systems in Europe because of its easy operation and minimum control (Vees et al., 2020). However, these Clostridia species are not able to hydrolyze lignocellulosic biomass efficiently (Maiti et al., 2018), so that sugar monomers need to be obtained in an upstream pretreatment stage, followed by hydrolysis. The pretreatment alters the complex polymeric biomass structure by breaking the lignin seal, removing lignin and/or increasing its porosity (García et al., 2011). The pretreatment should meet the following requirements: low energy demand and overall costs, efficient and rapid release of sugars in the subsequent hydrolysis, reducing carbohydrate degradation and avoiding the formation of inhibitory compounds (e.g., acids, furans and phenols). Concerning pretreatment of rice straw, several methods have been proposed, including: alkaline pretreatment (Moradi et al., 2013; Zhao et al., 2018; Zhu et al., 2005), acid pretreatment (Moradi et al., 2013; Ranjan and Moholkar, 2013; Zhao et al., 2018), steam explosion (Ranjan and Moholkar, 2013), organosolv pretreatment (Amiri et al., 2014) and the recent microwave-assisted hydrothermolysis (Valles et

al., 2020). Alkaline pretreatment with sodium hydroxide, ammonium hydroxide, potassium hydroxide or calcium hydroxide is effective in biomass with low lignin content and has other benefits such as low sugar degradation and a non-corrosive nature (Vivek et al., 2019). This chemical method disrupts and removes the lignin-carbohydrate structure and causes the biomass to swell, which reduces polymerization and cellulose crystallinity and increases the internal surface area. Among the different alkaline reagents, sodium hydroxide leads to a more efficient straw delignification (Hosseini et al., 2012), as well as other effects such as alteration of the silica layers and the cuticle wax, so that NaOH pretreatment could be proposed as the best method of increasing enzyme accessibility to cellulose in RS biomass (Imman et al., 2015; Kim and Han, 2012; Mukherjee et al., 2018). Indeed, Moradi et al. (2013) obtained a higher butanol production and overall conversion from RS pretreating with NaOH instead of H_2SO_4.

The operational conditions of the pretreatment affect the overall performance of butanol production, since they govern the susceptibility of the substrate to hydrolysis and the subsequent fermentation of the liberated sugars (Bajpai, 2016). The most common approach for optimizing pretreatment conditions such as temperature, time, reagent concentration and solid loading focuses on evaluating their effect on the hydrolysis performance or on the delignification degree. For example in RS pretreatment, Singh et al. (2014) used reducing sugar concentration as a response variable, Kim and Han (2012) employed glucose recovery from untreated biomass and Hosseini et al. (2012) and Mukherjee et al. (2018) adopted the quantity of lignin removed from the biomass. Nevertheless, RS hydrolysates with high glucose concentration it can lead to reduced efficiency in terms of butanol production, butanol yield and overall butanol productivity (Zhao et al., 2018).

Alternatively, the selection of a butanol to biomass ratio as response variable can be useful when pretreated hydrolysates are compared in terms of raw material conversion to butanol. The aim of this work was to optimize the alkaline pretreatment of RS considering the mass balance of the whole process from raw RS to butanol in order to assess the efficient use of lignocellulosic biomass. The global efficacy of the three serial steps: pretreatment, hydrolysis and ABE batch fermentation was defined by a single response variable. The effect of the temperature, time, NaOH concentration and solid loading was assessed on the (i) solid recovery, (ii) released sugars and (iii) produced butanol in order to integrate the efficiency of these three stages in the response variable, the butanol-biomass ratio (g butanol kg RS^{-1}). Pretreatment optimization was carried out by a preliminary fractional factorial design followed by a central composite design (CCD).

7.2. Materials and methods

7.2.1. Materials

RS was obtained from the Albufera Natural Park in Valencia (Spain). The raw material was milled and the particle size between 100 and 500 μm was selected by an ISO-3310.1 sieve (CISA, Spain). Before storing for further use, the biomass was dried in an oven at 45 °C to reduce residual moisture content below 5% w/w. The chemical composition (% dry weight basis) of the RS was: cellulose 35.8 ± 2.1%, hemicellulose 17.5 ± 1.4%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 14.3 ± 0.4%, ash 16.7 ± 0.1% and others 15.6 ± 3.7%.

Saccharification of the pretreated biomass was carried out by a commercial Cellic® CTec2 enzyme blend (Novozymes, Denmark). The cellulase activity resulted in a value of 157 filter paper units (FPU) mL^{-1} according to the National Renewable Energy Laboratory method (Adney and Baker, 1996). *Clostridium beijerinckii* DSM 6422 (NRRL B-592), obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was stored at -80 °C in a Reinforced Clostridial Medium with 20% (v/v) glycerol. Before ABE fermentation the cells were grown following the procedure described elsewhere (Valles et al., 2020).

7.2.2. Alkaline pretreatment and enzymatic hydrolysis

Alkaline pretreatment was carried out in 500-mL glass bottles in which the RS was mixed with an NaOH solution (concentration ranging from 0 to 2% w/v) to achieve the appropriate solid loading (5 or 10% w/ v), according to the experimental design described in Section 7.2.5. The bottles were then heated to 121 or 134 °C for a reaction time between 10 and 60 min in an autoclave (MED20, J.P. Selecta, Spain). The slurry was centrifuged at 4000 rpm for 6 min (Centrifuge 5804, Eppendorf, Germany), the solid phase was washed four times with deionized water and pH was adjusted to 6.5. Finally, the pretreated RS was dried at 45 °C for 24 h. The severity of the pretreatment conditions was estimated by the Severity Factor (SF) proposed by MacAskill et al. (2018):

$$SF = Log\left[time\ (min.) \times \exp\left[\frac{Temperature\ (^{\circ}C) - 100}{14.75}\right]\right] \quad (1)$$

Enzymatic hydrolysis was conducted in 100-mL conical flasks (with a working volume of 45 mL) containing 8% w/v of the pretreated RS to avoid end-product inhibition which was previously reported at higher RS loadings (Zhu et al., 2005).

The commercial enzyme blend Cellic® CTec2 was added with a load of 15 FPU g-dw^{-1} based on the optimal configuration obtained in our previous study on simultaneous saccharification and fermentation of microwave-pretreated RS (Valles et al., 2020). Hydrolysis was carried out at pH of 5.5 (50 mM acetate buffer), 50 °C and 200 rpm in an SI500 orbital shaker (Stuart, UK) for 72 h. After saccharification the samples were centrifuged at 4000 rpm for 10 min, filtered through 1.2 µm and stored at 4 °C until ABE fermentation.

7.2.3. ABE fermentation

Batch fermentations of 26 mL of the RS hydrolysate were performed in a 50 mL serum bottles with a working volume of 30 mL. The medium was composed of: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} NH_4Ac, 0.09 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001 g L^{-1} $MnSO_4 \cdot H_2O$, 0.02 g L^{-1} $FeSO_4 \cdot 7H_2O$ and 4 g L^{-1} of yeast extract. The initial pH was adjusted to 5.8, oxygen was displaced and bottles were autoclaved for 10 min at 121 °C. The bottles were inoculated with 2 mL (5% v/v) of *C. beijerinckii* DSM 6422, and incubated at 37 °C and 150 rpm for a maximum of 144 h.

7.2.4. Analytical methods

The efficiency of the pretreatment was characterized by analysis of sugars and inhibitory compounds from 1.5-mL samples of the RS hydrolysate. The chemical composition of the pretreated RS was determined for each pretreatment condition. Structural carbohydrates, lignin and ash were measured following the National Renewable Energy Laboratory procedures (Sluiter et al., 2008). Fermentation was monitored by analyzing pH, cell growth, products of acids and solvents, and sugar consumption from 1-mL samples withdrawn every 24 h. The pH was measured by a Minitrode electrode (Hamilton, USA). Cell density (g-dw L^{-1}) was determined from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany) and using the linear correlation: g-dw L^{-1} = $0.2153 \cdot OD_{600} + 0.0689$ (n = 10, R^2 = 0.9907).

Liquid samples were centrifuged at 10000 rpm for 5 min and filtered through 0.22 µm before HPLC analysis. Sugars (glucose, xylose and arabinose), acids (acetic acid, butyric acid and levulinic acid), solvents (butanol, acetone and ethanol) and other inhibitory compounds (furfural and 5-HMF) were analyzed by a liquid chromatograph (Agilent HPLC 1100 Series, Agilent Technologies, USA). An Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA) was operated at 50 °C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL min $^{-1}$. Sugars, butyric acid, butanol and ethanol were analyzed by a refractive index detector (RID).

A diode array detector (DAD) was used to measure acetic, formic and levulinic acids at 210 nm, while acetone, furfural and 5-HMF were analyzed at 280 nm. The total phenolic compounds were determined by the Folin-Denis method (Folin and Denis, 1912), expressing phenolic concentration as gallic acid equivalents (GAE).

For the evaluation of the enzymatic hydrolysis, the glucose yield was defined as:

$$Glucose\ yield\ (\%) = \frac{Glucose\ released\ \left(g\ L^{-1}\right) \times 0.9 \times 100}{\left[80\ \left(g\ L^{-1}\right)/Solid\ recovery\ (\%)\right] \times Cellulose\ in\ raw\ RS(\%)} \quad (2)$$

The overall conversion of RS into biobutanol or ABE solvents was calculated as follows:

$$Butanol\ (or\ ABE) \Big/ biomass\ ratio\ \left(g\ kg^{-1}\right) = \frac{Butanol\ (or\ ABE)\ produced\ (g)/V_{hydrolisate\ fermented}\ (L)}{\left[0.08\ \left(kg\ L^{-1}\right)/Solid\ recovery\ (\%)\right] \times 100} \quad (3)$$

7.2.5. Design of experiments and statistical analysis

The alkaline pretreatment of RS was optimized in two subsequent statistical analyses. In both cases, the butanol-biomass ratio (g kg RS^{-1}), which indicates the mass balance efficiency of the three serial stages (pretreatment, hydrolysis and ABE fermentation) was selected as the response parameter. First, the significant variables were selected in the overall conversion to butanol by fractional factorial design. These variables were then optimized by the response surface method using CCD. Design of experiments and data analysis were conducted using the MINITAB® v.2020.1.0 commercial software (Minitab Inc., USA). Analysis of variance (ANOVA) was performed at a confidence level of 95% (p-value < 0.05).

7.2.5.1. Fractional factorial design and data analysis

The significant factors affecting the butanol-biomass ratio at 72 h were screened by a 2^{4-1} fractional factorial design (resolution IV, 8 experiment runs). Table 7.1 summarizes the coded and real values of the four variables. Pretreatment temperature (X_1) and solid loading (X_4) were established as the categorical variables, while pretreatment time (X_2) and NaOH concentration (X_3) were the range variables.

Table 7.1. 2^{4-1} fractional factorial design of 4 variables.

	Independent variables	Coded and real values	
		Level -1	Level +1
X_1	Temperature (°C)[a]	121	134
X_2	Time (min)	10	40
X_3	NaOH concentration (% w/v)	0.2	2.0
X_4	Solid loading (% w/v)[a]	5	10

[a] Categorical

7.2.5.2. Central composite design and data analysis

Based on the results of the fractional factorial design, a response surface method with CCD (composed of 13 experiments with 5 central point replications) was used to determine the optimal combination of the significant variables (pretreatment time and NaOH concentration). The established range for each factor was as follows: pretreatment time (from 20 to 60 min) and NaOH concentration (from 0 to 1% w/v). Finally, a validation step was carried out by three replicates using the optimized conditions for RS use.

7.3. Results and discussion

7.3.1. Screening key factors on the overall conversion to butanol

The influence of the pretreatment variables (temperature, time, NaOH concentration and solid loading) was initially assessed for maximizing the release of the fermentable sugar and the butanol-biomass ratio. Table 7.2 summarizes the results of the fractional factorial design, including the solid recovery from pretreatment (%), sugars released after 72 h of enzymatic hydrolysis (g L^{-1}), butanol concentration (g L^{-1}), and butanol-biomass ratio (g kg untreated RS^{-1}) obtained from 72 h ABE fermentation. The alkaline pretreatment provided total solid recoveries ranging from 43.0 to 85.7%, with the highest values for the lowest NaOH concentration (runs 1 – 4) despite the SF.

Table 7.2. 2^{4-1} fractional factorial design matrix along with the values of SF, solid recovery (%), released sugars after 72 h-enzymatic hydrolysis (g L^{-1}), butanol production (g L^{-1}) and butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h of ABE fermentation.

Run	Real values				SF	Solid Recovery (%)	Released Sugars (g L^{-1})	Butanol (g L^{-1})	Butanol-Biomass ratio (g kg unt. RS^{-1})
	X_1[a]	X_2[b]	X_3[c]	X_4[d]					
1	121	10	0.2	5	1.62	78.4	17.3	2.8	24.7
2	134	10	0.2	10	2.00	83.4	10.4	1.7	16.4
3	121	40	0.2	10	2.22	85.7	4.9	0.3	2.5
4	134	40	0.2	5	2.60	72.3	33.4	5.2	42.9
5	121	10	2	10	1.62	55.4	43.8	4.6	29.3
6	134	10	2	5	2.00	44.0	61.2	4.4	22.2
7	121	40	2	5	2.22	43.0	65.3	10.6	51.9
8	134	40	2	10	2.60	48.6	46.1	2.1	11.4

[a] X_1: temperature (°C); [b] X_2: time (min); [c] X_3: NaOH concentration (% w/v); [d] X_4: solid loading (% w/v)

The influence of temperature, time and solid loading seems to be negligible on the partial biomass solubilization and/or degradation. Figure 7.1 shows the chemical composition (%) of raw and pretreated RS of the 8 runs. All pretreatment experiments with the highest NaOH concentration led to pretreated RS enriched in cellulose (>49%, runs 5 – 8), while a minimum time of 40 min was required to achieve some cellulose enrichment for the lowest NaOH concentration (40 – 42%, runs 3 – 4). The cellulose enrichment came mostly from the preferential removal of acid insoluble lignin rather than the hemicellulose solubilization, except for run 3, which hardly altered the biomass structure. The highest biomass loading of run 3 explains the lower release of sugars than in run 4 (4.9 vs. 33.4 g L^{-1}), these being unsuitable operational conditions for butanol production. High degrees of delignification (80.3 – 97.6%) were found in the experiments with the highest alkali concentration (2% w/v, runs 5 – 8), while moderate delignification (57.0%) was achieved in run 4 with the lowest alkali concentration (0.2% w/v).

The highest cellulose recovery (84.1%) was from the soft alkaline conditions (run 4), which positively influenced the subsequent hydrolysis and fermentation processes. Alkaline pretreatment is known to be able to remove lignin from RS by producing pores on its surface (Mukherjee et al., 2018). Other pretreatments such as acid or microwave-assisted hydrothermolysis resulted in lower delignification degrees, which could limit enzymatic sugar recovery to some extent. Moradi et al. (2013) obtained a 27% delignification from acid pretreatment (50 °C, 30 min and 85% H_3PO_4) and Valles et al. (2020) obtained 13.3% delignification by microwave-

assisted hydrothermolysis at a ramp temperature from 100 °C to 200 °C for 40 min. The delignification achieved in this study was better than those obtained with other RS alkaline pretreatments. For example, Kim and Han (2012) achieved less than 80% delignification working at a longer reaction time (60 min) and lower temperature (100 °C). Mild alkaline conditions can also achieve high delignification but they require a high NaOH concentration. Moradi et al. (2013) reported 76% delignification working at 0 °C, 3 h and 12% w/v NaOH.

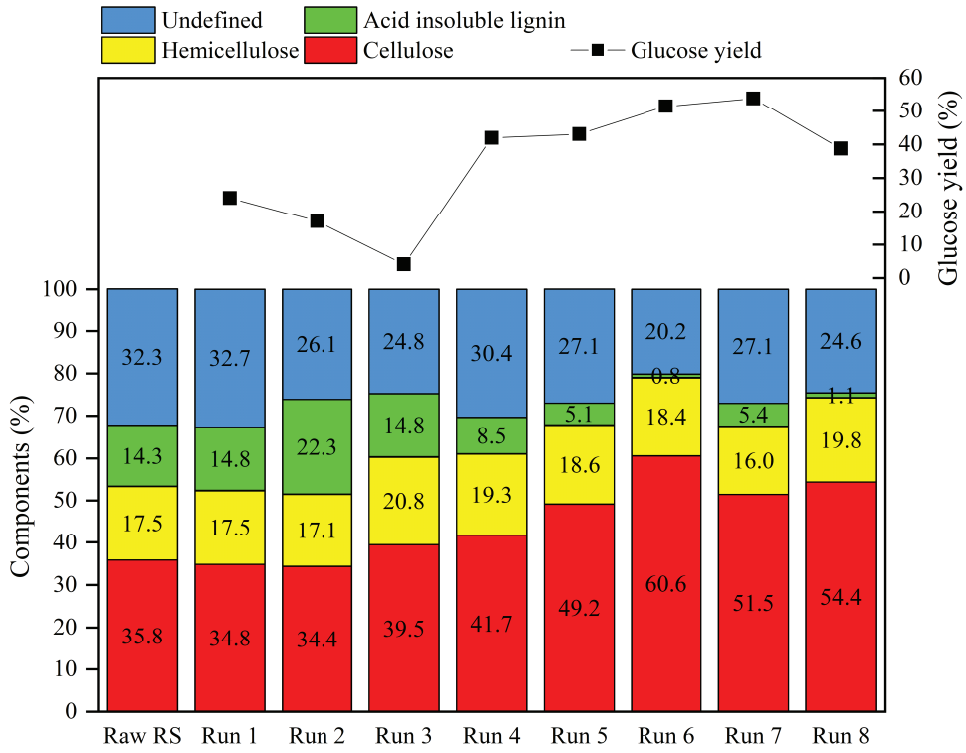

Figure 7.1. Chemical composition of raw RS and RS after the different pretreatments included in the 2^{4-1} fractional factorial design with glucose yield (%, referred to untreated RS) after 72 h of enzymatic hydrolysis.

The sugars released after 72 h of enzymatic hydrolysis of the pretreated samples confirmed the efficiency of alkaline RS pretreatment. The three samples (runs 1 – 3) with delignification degrees less than 20% provided the lowest sugar concentrations (<18 g L^{-1}, Table 7.2), since lignin hinders cellulase access to cellulose fibers (Kim and Han, 2012) and binds non-productively to cellulase (Lan et al., 2013). The maximum sugar concentration was obtained in run 7, reaching a value of 65.3 g L^{-1} (50.5 g L^{-1} glucose, 13.5 g L^{-1} xylose and 1.3 g L^{-1} arabinose) although this experiment did not lead to the highest delignification. This could be attributed to the lower hemicellulose content of this sample (16.0%, Figure 7.1), as hemicellulose can inhibit hydrolysis since acetyl groups from xylan cause steric hindrance of enzymes (Kong et al., 1992). Nevertheless, these results showed that non-specific adsorption of the enzyme to the remained lignin do not play an adversely effect.

The results of glucose yield (Figure 7.1) showed that by increasing SF and reducing the solid loading the consumption of NaOH can be reduced to achieve similar values (runs 4 and 5 resulted in glucose yields ~40%). Hydrolysates were measured to quantify the potential inhibitory compounds. The analysis outcomes showed that the viability of the subsequent fermentation would not be negatively affected by the presence of inhibitory compounds. Levulinic acid and furfural were not detected in any of the samples and HMF concentration was negligible (<0.01 g L^{-1}). Total phenolic compound concentration ranged from 0.14 to 0.32 g L^{-1}, below the inhibitory level (0.71 g L^{-1}) for *C. beijerinckii* DSM 6422 (López-Linares et al., 2019). The acetic acid in the hydrolysate was mainly formed during alkaline pretreatment by hydrolysis of the acetyl groups of hemicellulose (Jönsson et al., 2013). The concentration of acetic acid ranged from 2.58 to 4.45 g L^{-1}, which could be beneficial for ABE fermentation as it has been reported that 3 g L^{-1} of acetic acid enhanced solvent production by *C. beijerinckii* DSM 6422 (Bellido et al., 2015).

The hydrolysates were fermented by *C. beijerinckii* DSM 6422 to assess the efficiency of the whole process. As can be seen in Table 7.2, maximum concentration (10.6 g L^{-1}) was obtained for the maximum released sugars (65.3 g L^{-1}, run 7). In terms of sugar conversion to butanol, run 4 achieved similar values to run 7 (0.16 g butanol g released $sugars^{-1}$) in spite of its lower sugar concentration. The lower butanol production of runs 5, 6 and 8 was related to poor sugar conversion. After 24 h of fermentation, undissociated acids were higher in those samples than in run 7 (acetic and butyric acid: 23.0 ± 1.3 mM in runs 5, 6 and 8; 16.0 mM in run 7). Undissociated acids can pass across the *Clostridium* cell membrane, while their subsequent dissociation at the neutral internal pH of the cell can result in growth inhibition (Jones and Woods, 1986). To keep the undissociated acid concentration at < 16 mM, initial pH was set at 5.8 for further experiments. RS-to-butanol conversion was analyzed to consider the loss of solids along with the sugar conversion (Table 7.2). The highest RS use was achieved in run 7 with a butanol-biomass ratio of 51.9 g kg untreated RS^{-1}, followed by run 4 with a value of 42.9 g kg untreated RS^{-1}. Both experiments had the lowest solid loading (5% w/v) but different NaOH requirements. Valles et al. (2020) pretreated rice straw by microwave assisted hydrothermal hydrolysis, obtaining 51 g kg untreated RS^{-1} as the best value, showing that further optimization of alkaline pretreatment would be promising for efficient RS use.

The ANOVA regression model of the butanol-biomass ratio at 72 h (Table 7.2) was statistically significant with a p-value lower than 0.05 (95% confidence, p-value of 0.0479). Among the four variables included in the 2^{4-1} fractional factorial design, the solid loading (p-value of 0.0205) and the interaction between temperature and

NaOH concentration (p-value of 0.0218) were found to be significant with a negative effect on the butanol-biomass ratio (g kg RS^{-1}). The good accuracy of the model was due to the coefficient of determination (R^2: 0.9805) and adjusted coefficient of determination (Adj. R^2: 0.9319) values. Considering the negative effect of the solid loading (categorical parameter) on the process efficiency the value of 5% w/v was selected for further optimization. For this solid loading, a model prediction was carried out to select the temperature level (categorical parameter) and the range of NaOH concentration and time to define the CCD. Figure 7.2 shows the cube plot of fitted means of the butanol-biomass ratio, in which the vertices of the cube display the model results of the predicted values for all the combinations of the low and high levels of three parameters (temperature, NaOH concentration and time). As can be seen, there are two sets of temperature-NaOH conditions with RS-to-butanol conversion values higher than 40 g kg RS^{-1}. The two vertices lie on the plane intersecting the cube at 40 min corresponding to the pairs: 134 °C with 0.2% w/v NaOH and 121 °C with 2.0% w/v NaOH. Due to the potential saving in the reagent cost, it was decided to use 134 °C as the working temperature. From the edge at 40 min and 134 °C, it was decided to limit the maximum NaOH amount to 1% w/v.

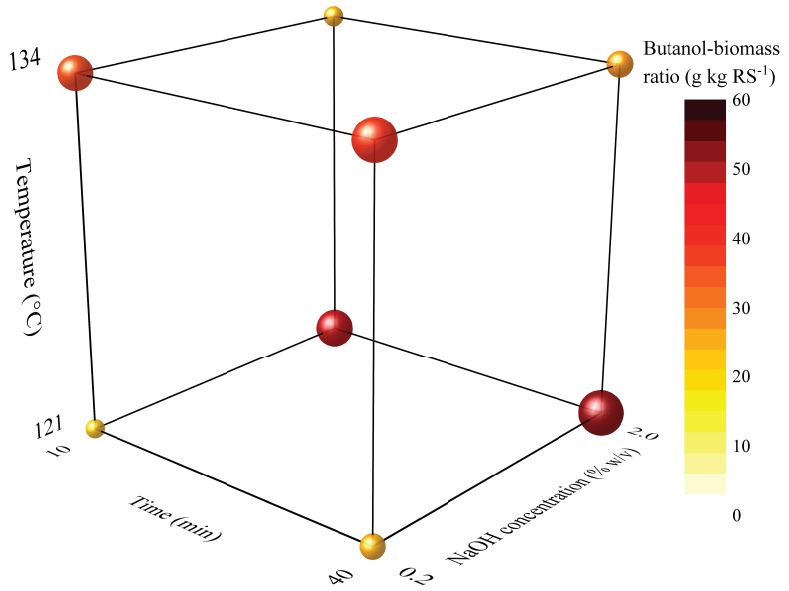

Figure 7.2. 2^{4-1} fractional factorial cube plot using predicted model values of butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h. Solid loading = 5% w/v.

7.3.2. Optimization of butanol-biomass ratio

Once the temperature and solid loading were set at 134 °C and 5% w/v from the results of the preliminary fractional factorial design experiment, a response surface method with full factorial CCD was carried out. The maximum butanol-biomass ratio was found from the best NaOH concentration and time conditions. To

confirm the goodness of the optimization approach, the model was validated by running an experiment (3 replicates) in the optimum conditions.

7.3.2.1. Response surface methodology

The RS-to-butanol conversion was maximized through a five-level, two-factor CCD, followed by linear regression analysis to adjust the experimental values to a second-order model. The experimental design is shown in Table 7.3 and includes the coded and real values of the range factors (NaOH concentration: Z_1; time: Z_2) for the 13 experimental runs, including five central point replications to assess the experimental variability. The central point conditions (0.5% w/v of NaOH concentration and 40 min pretreatment time) were selected from previous results. The axial point conditions ($\alpha = 1.4142$) were set to extend the NaOH concentration from 0 (hydrothermal equivalent) to 1% w/v with time in the range ± 20 min. As temperature was set at 134 °C, a narrower SF variation (2.30 – 2.78) was used than in the previous factorial design (1.62 – 2.60). Table 7.3 also summarizes the response for the butanol-biomass ratio (g kg untreated RS^{-1}) along with the results of the three serial processes: (i) solid recovery (%) from alkaline pretreatment; (ii) released sugars (g L^{-1}) and glucose and xylose yields of untreated RS (%) in enzymatic hydrolysis; and (iii) butanol produced (g L^{-1}) after 72 h of ABE fermentation.

Table 7.3. CCD experimental matrix along with the values of solid recovery (%), released sugars (g L^{-1}) and glucose and xylose yield (%, referred to untreated RS) after 72 h-enzymatic hydrolysis, butanol production (g L^{-1}) at 72 h and the observed and predicted values of butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Run	Coded values		Real values		Solid recovery (%)	Released sugars (g L^{-1})
	Z_1[a]	Z_2[b]	Z_1[a]	Z_2[b]		
1	-1	-1	0.15	26	71.8	17.0
2	+1	-1	0.86	26	44.4	56.9
3	-1	+1	0.15	54	68.4	24.4
4	+1	+1	0.86	54	41.7	57.3
5	-α[c]	0	0.00	40	75.9	8.6
6	+α[c]	0	1.01	40	37.9	64.4
7	0	-α[c]	0.50	20	51.4	51.1
8	0	+α[c]	0.50	60	47.6	54.8
9	0	0	0.50	40	50.6	50.8
10	0	0	0.50	40	50.6	53.6
11	0	0	0.50	40	50.6	51.1
12	0	0	0.50	40	50.5	53.1
13	0	0	0.50	40	50.6	51.1

[a] Z_1: NaOH concentration (% w/v); [b] Z_2: time (min); [c] $\alpha = 1.4142$

Good reproducibility of the representative parameters was obtained for the three stages of the process for the central point replicates (run 9 – 13; solid recovery: 50.6 ± 0.0%; released sugars: 51.9 ± 1.3 g L^{-1}; butanol production: 8.8 ± 0.5 g L^{-1}). A broad solid recovery range was achieved from the experimental results (37.9 – 75.9%), which negatively correlated with the cellulose content of the samples. Of the two tested parameters, NaOH concentration had a strong effect, with RS pretreatment being less sensitive to time changes. This is in agreement with the results that Mukherjee et al. (2018) obtained from alkaline pretreatment with a wider range of NaOH concentrations (0.73 – 12.73% w/v), time (39.55 – 140.45 min) and temperature (16.45 – 133.86 °C). They found that alkali concentration was more important in RS delignification during pretreatment than the other two factors. Especially when alkali concentration was as low as 0.15% w/v (runs 1, 3) or when alkali was not used (run 5) small solid losses were achieved, thus indicating the inefficiency of mild alkali usage for increasing the sugar released from RS. Indeed, these samples had a similar lignin content (13.2 – 16.9%) to the raw material. Insoluble lignin was extensively removed for 0.5% w/v NaOH or higher, while the content in the pretreated solid fractions varied from 0.7 to 6.4% (runs 2, 4, 6, 7 – 13). These findings show that similar RS delignification can be achieved by limiting NaOH concentration to a maximum of 1% w/v as by using 2% w/v NaOH (factorial fraction screening, Figure 7.1).

Table 7.3. cont. CCD experimental matrix along with the values of solid recovery (%), released sugars (g L^{-1}) and glucose and xylose yield (%, referred to untreated RS) after 72 h-enzymatic hydrolysis, butanol production (g L^{-1}) at 72 h and the observed and predicted values of butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Glucose yield (%)	Xylose yield (%)	Butanol (g L^{-1})	Butanol-biomass ratio (g kg untreated RS^{-1})	
			Observed	Predicted
23.0	32.9	3.3	34.2	27.4
55.6	50.4	10.3	66.0	67.4
31.7	43.7	4.8	46.9	39.5
53.3	46.4	10.2	61.3	62.1
11.6	20.7	0.0	0.0	8.9
54.9	46.7	10.2	55.9	53.1
55.7	56.8	8.4	62.1	64.7
57.7	51.8	9.6	66.1	69.6
56.5	52.8	8.1	58.9	64.4
59.7	54.0	9.5	69.2	64.4
56.7	53.3	8.7	63.6	64.4
58.2	55.3	9.0	65.6	64.4
56.0	53.7	8.9	64.7	64.4

All the pretreated solid fractions without any further detoxification were enzymatically hydrolyzed. As can be seen in Table 7.3, the concentration of the reducing sugars in the hydrolysate increased mainly with NaOH concentration, reaching the maximum value of 64.4 g L^{-1} (46.2 g L^{-1} glucose, 16.5 g L^{-1} xylose and 1.7 g L^{-1} arabinose) for the highest NaOH concentration (1% w/v, run 6), and the minimum value of 8.6 g L^{-1} (4.8 g L^{-1} glucose, 3.7 g L^{-1} xylose and 0.1 g L^{-1} arabinose) in the absence of NaOH (run 5). When low lignin removal was obtained (runs 1, 3 and 5), glucose yield varied from 11.6 to 31.7% and xylose yield varied from 20.7 to 43.7%. In the experiments with a high degree of delignification (runs 2, 4, 6, 7 – 13) more sugars were recovered from raw RS (53.3 – 59.7% for glucose and 46.4 – 56.8% for xylose). Although the experiments with high delignification lost more sugars, higher yields were obtained as enzymatic digestibility was improved. The glucose/xylose ratio in these experiments (2.6 ± 0.1) was substantially higher than the samples with NaOH concentration ≤0.15% w/v (1.6 ± 0.2). Solubilization of hemicellulose during pretreatment occurred at low NaOH doses. However, compared with the results of the fractional factorial screening (Table 7.1), it was confirmed that 1% w/v NaOH would be enough to reach the maximum concentration of fermentable sugars (~65 g L^{-1}).

The hydrolysates were subsequently fermented and the butanol production was evaluated. The low sugar content of run 5 made solvent production unfeasible as the bacteria metabolism did not have enough carbon sources to assimilate the acids produced during the acidogenic phase. In the rest of the samples, the ABE fermentation was not negatively impacted by the potentially inhibitory compounds produced during pretreatment. Levulinic acid and furfural were not detected in the hydrolysate and the concentrations of HMF (<0.01 g L^{-1}), while total phenolic compounds (0.31 ± 0.08 g L^{-1}) and acetic acid (3.95 ± 0.80 g L^{-1}) were below the inhibitory threshold level. Interestingly, the use of 0.15% w/v NaOH (runs 1 and 3) provided the highest butanol conversion of the released sugars (0.20 g butanol g released $sugars^{-1}$). Zhang et al. (2014) reported that an acid insoluble lignin content of 8.55% in alkaline-pretreated corn stover by twin-screw extrusion released up to 0.74 g L^{-1} of soluble lignin compounds which inhibited ABE fermentation by *C. acetobutylicum* ATCC 824. In contrast, our results indicate that the lower lignin removal rate during RS pretreatment does not seem to have a negative impact on the Clostridia metabolism. The lack of by-product inhibition in our work could be attributed to the differences in the lignocellulosic biomass combined with the differences in the alkaline application. All the samples with a high lignin removal rate (runs 2, 4, 6 – 13) from the abundant carbon source in the hydrolysates (>50 g L^{-1}) produced high butanol concentrations (8.1 – 10.3 g L^{-1}), although the higher

sugar concentration was not accompanied by higher sugar-to-butanol conversion. Although run 6 achieved the maximum sugar concentration (64.4 g L^{-1}), the sugar-to-butanol conversion was the lowest (0.16 g butanol g released $sugars^{-1}$), due to the lower xylose uptake than in the samples with sugar concentrations in the range of 51 – 57 g L^{-1}. This could be attributed to the greater impact of the carbon catabolite repression phenomena for glucose levels of ~65 g L^{-1} (Zhao et al., 2018). These results show the importance of assessing the effectiveness of the pretreatment process by not only evaluating the amount of fermentable sugar released after hydrolysis but also the butanol conversion from fermentation.

The three-stage butanol-biomass ratio was therefore selected as the variable response for maximizing RS use. Regardless of the pretreatment reaction time, this ratio varied from 55.9 to 69.2 g kg untreated RS^{-1} when NaOH concentration was 0.5% w/v or higher (runs 2, 4, and 6 – 13), which is a considerable improvement on the previous fractional factorial screening (maximum value of 51.9 g kg untreated RS^{-1}, Table 7.1). The quadratic model obtained for the butanol-biomass ratio versus NaOH concentration (Z_1) and time (Z_2) is described by Equation 7.4.

$$Butanol - biomass\ ratio = -2.9 + 212.8Z_1 - 0.00Z_2 - 132.5Z_1^2 + 0.0071Z_2^2 - 0.871Z_1Z_2 \quad (4)$$

The ANOVA and the coded regression coefficients of the quadratic model are summarized in Table 7.4. The results of the statistical analysis showed that the model was highly significant at the confidence levels (95%, p-value = 0.0004), whereas the lack-of-fit was not significant (p-value = 0.0777). The value of the coefficient of determination (R^2) was 0.9389, indicating that the experimental results had a good correlation with the predicted ones (Table 7.3), in which only 6.11% of the total variations were not explained by the fitted model. The value of the adjusted coefficient of determination (Adj. R^2: 0.8953) confirmed that Equation 7.4 can adequately describe the effect of NaOH concentration and time on the butanol-biomass ratio obtained from RS after 72 h. The NaOH concentration had a much higher influence than time on the response. Both the linear (Z_1) and the quadratic (Z_1Z_1) coefficient were found to be significant with the same p-value (0.0002) but with opposite effects, while neither the linear (Z_2, p-value of 0.4584) or the quadratic (Z_2Z_2, p-value of 0.5725) coefficients of time were significant. The interaction effect between both factors was also found to be insignificant (Z_1Z_2, p-value of 0.2028).

Table 7.4. ANOVA of the CCD model for butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Source	Degrees of freedom	Sum of squares	Mean square	F value	p-value Prob > F	Coeffi-cient[a]
Model	5	4088.61	817.72	21.52	0.0004	
Linear	2	1985.18	992.59	26.12	0.0006	
Z_1: NaOH concentration	1	1961.79	1961.79	51.63	0.0002	15.66
Z_2: time	1	23.39	23.39	0.62	0.4584	1.71
Square	2	2028.43	1014.21	26.69	0.0005	
Z_1Z_1	1	1938.68	1938.68	51.02	0.0002	-16.69
Z_2Z_2	1	13.32	13.32	0.35	0.5725	1.38
2-way interactions	1	75.00	75.00	1.97	0.2028	
Z_1Z_2	1	75.00	75.00	1.97	0.2028	-4.33
Error	7	265.98	38.00			
Lack-of-fit	3	209.72	69.91	4.97	0.0777	
Pure error	4	56.26	14.07			
Total	12	4354.58				
Standard Deviation, SD					6.1642	
R^2					0.9389	
Adj. R^2					0.8953	

[a] For coded variables

The model was used to plot the three-dimensional response surface and the associated two-dimensional contour to map the optimal combination of the evaluated factors (Figure 7.3 a). The butanol-biomass ratio increases as NaOH concentration rises from 0 to 0.5% w/v, until reaching a saddle-shaped region. From 0.8% w/v NaOH, the response decreases as alkali concentration increases. The contour plot shows the weak interaction between NaOH concentration and time. Indeed, there are two opposing time values that led to the highest RS-to-butanol conversion: ~20 – 25 min or ~55 – 60 min for very similar NaOH concentrations (0.6 – 0.8% w/v). Of these two zones, the shorter time is the best cost-wise option, since the additional reagent is negligible relative to the extra time required. The model estimated the maximum butanol-biomass ratio at 72 h of fermentation (71.9 g kg untreated RS^{-1}) with 0.75% w/v NaOH and 20 min pretreatment time.

The combined effect of NaOH concentration and time on solid recovery after pretreatment (Figure 7.3 b), sugars released in enzymatic hydrolysis (Figure 7.3 c) and butanol produced in fermentation (Figure 7.3 d) were also plotted. Solid losses increase gradually with NaOH concentration due to the higher solubilization of the biomass in harsh alkaline conditions (Figure 7.3 b). The amount of fermentable sugar in the hydrolysate predicted to increase with NaOH concentration in the

pretreatment rises to the optimal value (Figure 7.3 c), which was estimated by the model to be 61.7 g L^{-1} for an NaOH concentration of 0.88% w/v (and a pretreatment time of 39 min). As expected, both contour plots show very flat profiles versus time. The response surface plot for butanol production (Figure 7.3 d) shows a saddle shape similar to that of the butanol-biomass ratio (Figure 7.3 a), with two optimal regions near the minimum and maximum tested times ~20 – 23 min and ~55 – 60 min, but for higher NaOH dose (~0.9% w/v). Indeed, the butanol production quadratic model predicted a maximum butanol concentration of 10.6 g L^{-1} using 0.88% w/v NaOH and 21 min alkaline pretreatment.

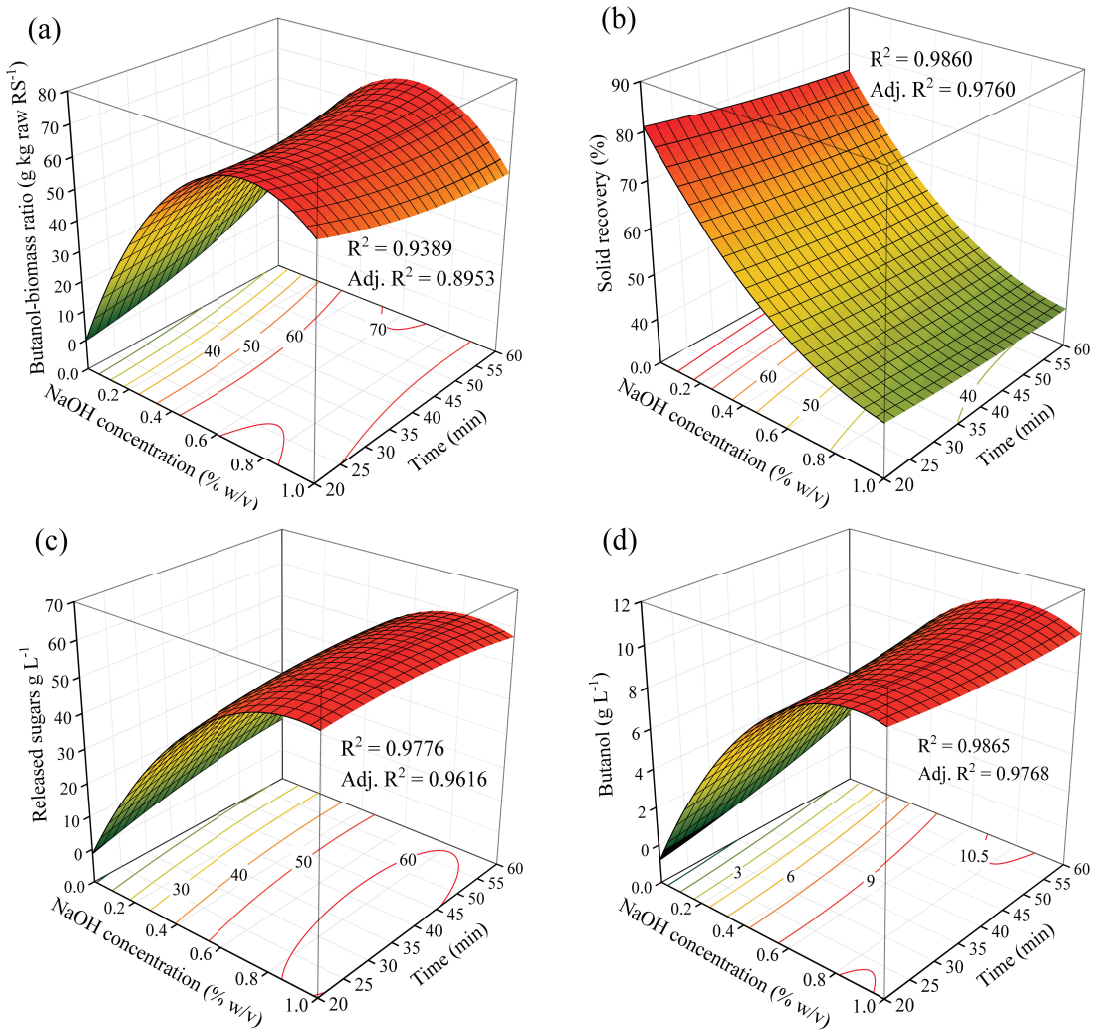

Figure 7.3. Response surface and corresponding contour plot for (a) butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h, (b) solid recovery (%), (c) released sugars (g L^{-1}) after 72 h-enzymatic hydrolysis and (d) butanol production (g L^{-1}) at 72 h: combined effect of NaOH concentration (% w/v) and time (min).

The discrepancies in alkali severity between butanol titer and RS-to-butanol conversion are attributed to the different extension of the solubilization of the structural components of the biomass during pretreatment. Other authors have also

found that larger amounts of butanol and ABE can lead to lower mass balance efficiency (Amiri et al., 2014; López-Linares et al., 2020). Our results show the importance of selecting the right variable response during process optimization. The alkali dosage which maximizes fermentable sugars correlates well with the maximum butanol titer, but underestimates RS use by 13%. To improve the mass balance efficiency of ABE fermentation from the lignocellulosic biomass, the efficiency of all three pretreatment, saccarification and fermentation stages should be jointly considered when optimizing the operational parameters of the pretreatment process.

7.3.2.2. Model validation

The validation of the optimum value for the second-order model of the butanol-biomass ratio was carried out in triplicate using 5% w/v RS pretreated with 0.75% w/v NaOH at 134 °C for 20 min. The following profiles are depicted in Figure 7.4: pH, cell density, sugar concentration (glucose, xylose and arabinose), acid concentration (acetic and butyric acid) and solvent concentration (acetone and butanol, ethanol was not detected). A ~12 h lag phase was observed, after which the *Clostridium* culture started to grow by consuming glucose, indicating its adaptation to the nutritional environment. The exponential phase of cell growth was reached at ~24 h, although there was almost no acid accumulation or a notable change in pH shift in the first 24 h.

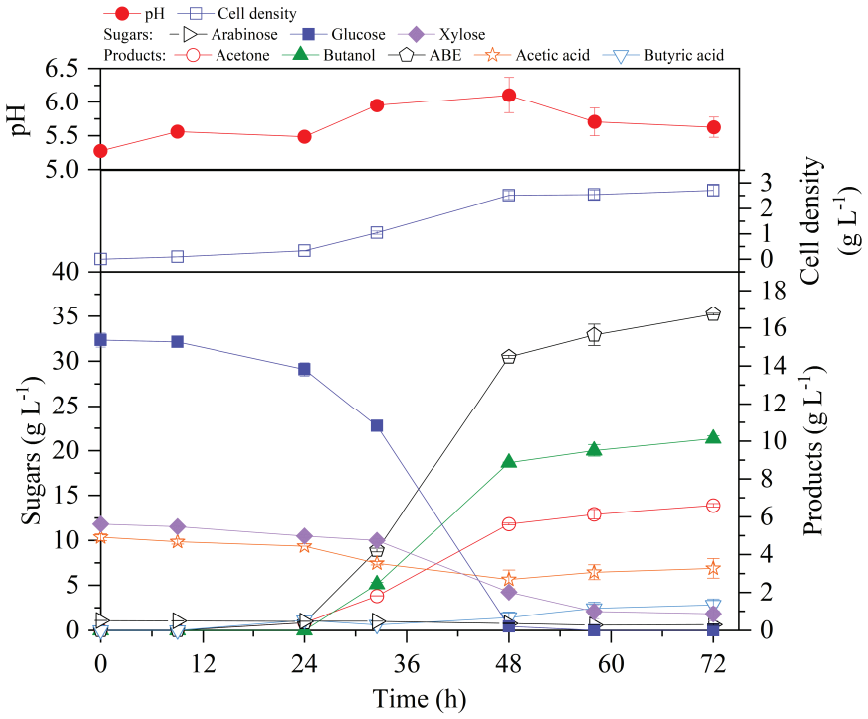

Figure 7.4. CCD model validation at the predicted optimum conditions.

The absence of a substantial drop in pH during sugar consumption (acidogenesis stage) was related to the initial amount of acetic acid from the RS hydrolysate (4.9 ± 0.2 g L^{-1}). The acetic acid enhanced the early development of a mixed population of solvent and acidogenic cells with the coupled production of acids and solvents (Chen et al., 2013; Maddox et al., 2000). In this case, the early production of butanol and acetone as an acid consumer step caused a slight increase in pH from 24 to 33 h. Interestingly, 87% of the butanol (8.9 ± 0.1 g L^{-1}) and ABE solvents (14.5 ± 0.1 g L^{-1}, butanol:acetone mass ratio of 1.58) were produced in 48 h, accompanied by almost complete glucose depletion and about two-thirds of the xylose uptake, giving maximum butanol and ABE productivities of 0.185 ± 0.001 g L^{-1} h^{-1} and 0.302 ± 0.002 g L^{-1} h^{-1} respectively. Maximum butanol (10.1 ± 0.2 g L^{-1}) and ABE (16.7 ± 0.1 g L^{-1}, butanol:acetone mass ratio of 1.54) concentrations were obtained at the end of fermentation (72 h), with a butanol yield of 0.24 ± 0.01 g g^{-1} and ABE yield of 0.39 ± 0.01 g g^{-1}. In contrast to our results, production of similar butanol concentrations from non-detoxified NaOH-pretreated lignocellulosic biomass hydrolysates is related to an extensive fermentation time. For example, Cai et al. (2016) achieved 9.4 g L^{-1} of butanol and 12.2 g L^{-1} of ABE from corn cob after 60 h of fermentation by *C. acetobutylicum* ABE 1301. Fernández-Delgado et al. (2019) reported 11.3 g L^{-1} of butanol and 14.5 g L^{-1} of ABE after 96 h of fermentation from brewer's spent grain by *C. beijerinckii* DSM 6422. Only Gao and Rehmann (2014) achieved high solvent titers (12.3 g L^{-1} of butanol and 19.4 g L^{-1} of ABE) in short fermentation times (36 h) with NaOH-pretreated corn cob using *C. saccharobutylicum* DSM 13864. Our results indicate that RS alkaline pretreatment without further detoxification can achieve high butanol production accompanied by a high productivity. These results are promising for a further scale-up, as butanol productivity and not butanol concentration is the variable response of interest for *in-situ* butanol recovery processes (Ezeji et al., 2007). In these integrated processes enhancing butanol productivity is critical to improving the butanol removal rate (Moon et al., 2011).

A butanol-biomass ratio of 77.6 ± 1.1 g kg untreated RS^{-1} was achieved at the end of fermentation with a solid recovery after pretreatment of 53.0 ± 0.1%. The observed value was slightly higher than the predicted one (71.9 g kg untreated RS^{-1}), validating the proposed model on butanol-biomass ratio. The discrepancy between the experimental and predicted values was 7.3%, which nearly matched the predicted deviation from the value of R^2 (6.1%), and was even better than that predicted from Adj. R^2 (adjusted coefficient of determination, 11.5%). Valles et al. (2020) pretreated the same waste by microwaves and obtained a butanol-biomass ratio one-third lower (51 g kg untreated RS^{-1}) due to biomass delignification being

less efficient than alkaline pretreatment. Few studies to date have assessed the overall lignocellulosic biomass conversion to butanol. Neither are there any previous reports on using the global mass balance efficiency of the three sequential steps: pretreatment, hydrolysis and fermentation, as the variable response in optimization. The RS converted to butanol achieved in the present study was quite similar to that obtained by Amiri et al. (2014), 80 g butanol kg untreated RS^{-1} with an ethanol organosolv pretreatment, while a higher value (96 g butanol kg untreated RS^{-1}) was reported by Chi et al. (2019) with NaOH-pretreatment. However, the high butanol-biomass ratios of these studies were not accompanied by high butanol titers (about 5 – 6 g L^{-1}). Our butanol production was nearly double and also achieved higher maximum productivity. It is important to note that the butanol stripping rate increases when the butanol titer in the reactor increases due to the enhanced butanol mass transfer associated with its higher driving force (Xue et al., 2014). The promising mass balance efficiency, butanol titer and productivity achieved here with alkaline-pretreated RS shows promise for further research on reducing the cost by combining several stages in a single reactor (simultaneous saccharification and fermentation; simultaneous saccharification, fermentation and an *in-situ* recovery process).

7.4. Conclusions

Pretreating rice straw with NaOH was optimized to enhance rice straw conversion to butanol in batch ABE fermentation. Using the butanol-biomass ratio as the response variable has been identified as the key decision, since maximizing the sugar release without taking fermentation into account leads to underestimating RS use. By selecting the optimal conditions, the RS-to-butanol conversion was 77.6 g kg untreated RS^{-1} and a butanol titer as high as 10.1 g L^{-1} was achieved. In future studies, simultaneous saccharification and fermentation coupled with integrated recovery process will be carried out to increase the butanol productivity and final titer.

Acknowledgements

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Appendix A. Supplementary data

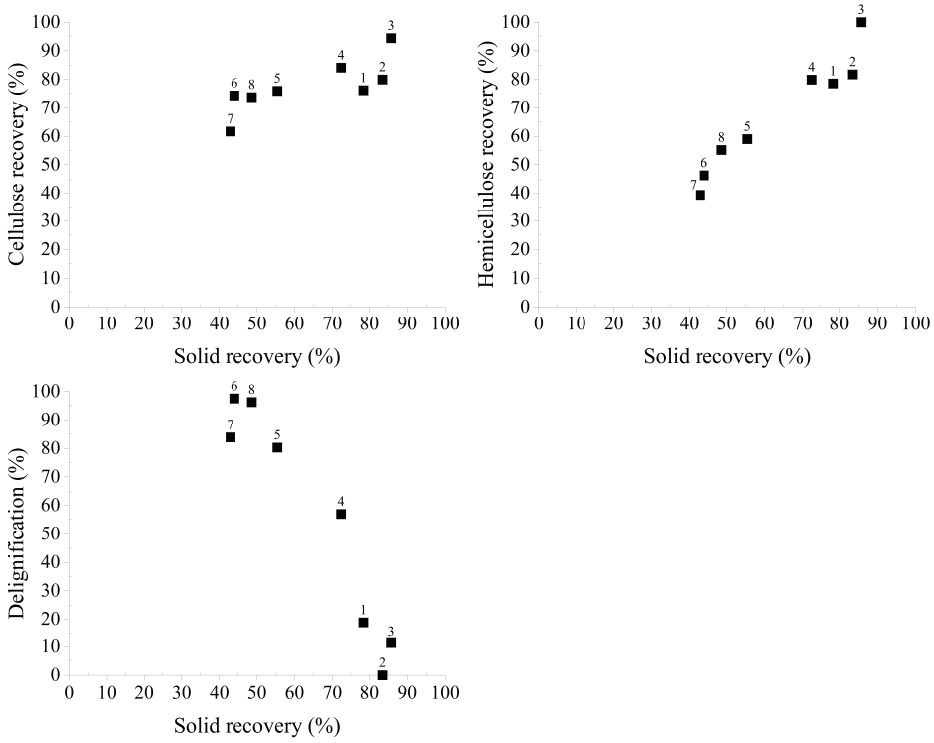

Figure 7.S1. CCD model validation at the predicted optimum conditions.

Table 7.S1. ANOVA of the 2^{4-1} fractional factorial design model for butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Source	Degrees of freedom	Sum of squares	Mean square	F value	p-value Prob > F	Coeffi-cient[a]
Model	5	1799.08	359.82	20.16	0.0479	
Linear	4	1006.46	251.61	14.09	0.0673	
X_1: temperature (°C)	1	29.99	29.99	1.68	0.3243	-3.87
X_2: time (min.)	1	32.28	32.28	1.81	0.3109	4.02
X_3: NaOH concentration (% w/v)	1	100.61	100.61	5.64	0.1409	7.09
X_4: solid loading (% w/v)	1	843.58	843.58	47.26	0.0205	-20.54
2-way interactions	1	792.62	792.62	44.4	0.0218	
X_1X_3	1	792.62	792.62	44.4	0.0218	-19.91
Error	2	35.7	17.85			
Total	7	1834.78				
Standard Deviation, SD					4.2251	
R^2					0.9805	
Adj. R^2					0.9319	

[a] For coded variables

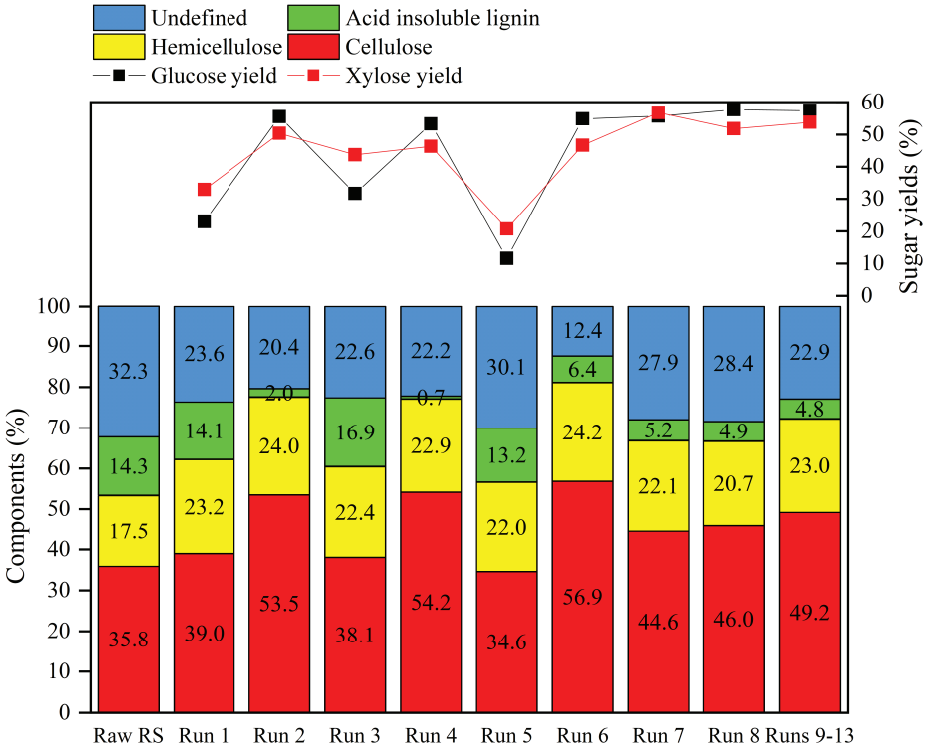

Figure 7.S2. Chemical composition of raw RS and RS after the different pretreatments included in the CCD with glucose and xylose yield (%, referred to untreated RS) after 72 h of enzymatic hydrolysis.

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8. FED-BATCH SIMULTANEOUS SACCHARIFICATION AND FERMENTATION INCLUDING *IN-SITU* RECOVERY FOR ENHANCED BUTANOL PRODUCTION FROM RICE STRAW

FED-BATCH SIMULTANEOUS SACCHARIFICATION AND FERMENTATION INCLUDING *IN-SITU* RECOVERY FOR ENHANCED BUTANOL PRODUCTION FROM RICE STRAW

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Abstract

This paper describes a study of fed-batch SSFR (simultaneous saccharification, fermentation and recovery) for butanol production from alkaline-pretreated rice straw (RS) in a 2-L stirred tank reactor. The initial solid (9.2% w/v) and enzyme (19.9 FPU g-dw^{-1}) loadings were previously optimized by 50-mL batch SSF assays. Maximum butanol concentration of 24.80 g L^{-1} was obtained after three biomass feedings that doubled the RS load (18.4% w/v). Butanol productivity (0.344 g L^{-1} h^{-1}) also increased two-fold in comparison with batch SSF without recovery (0.170 g L^{-1} h^{-1}). Although fed-batch SSFR was able to operate with a single initial enzyme dosage, an extra dosage of nutrients was required with the biomass additions to achieve this high productivity. The study showed that SSFR can efficiently improve butanol production from a lignocellulosic biomass accompanied by the efficient use of the enzyme.

Highlights

- Fed-batch SSFR is an efficient configuration to produce butanol
- A biomass load of 18.46% (w/v) was quickly processed to 24.80 g L^{-1} of butanol
- High productivity of 0.344 g L^{-1} h^{-1} was achieved
- One enzyme dosage improved the economic viability of the process

Keywords

Butanol; fed-batch; gas stripping; rice straw; simultaneous saccharification and fermentation

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8.1. Introduction

Global warming, one of the greatest challenges facing world, is accelerating the transformation of the energy system. The current EU policy includes the production of biofuels from biomass wastes as one of the systems to achieve a successful climate-neutral transition (European Union: European Commission, 2020). Compared to other liquid biofuels such as ethanol, biobutanol has a higher energy density and is less hygroscopic and corrosive (Schubert, 2020). Butanol production by ABE fermentation from agricultural waste (rice straw, wheat straw or sugarcane bagasse among others) has been explored in the last decade (Abo et al., 2019; Vees et al., 2020). Although these lignocellulosic residues are an abundant and low-cost feedstock, they require pretreatment and saccharification prior to fermentation. In the case of rice straw (RS), which has a much higher ash and silica content than other agricultural lignocellulosic by-products (Satlewal et al., 2018), the pretreatment method should be selected considering that these components hinder accessibility to inner cellulose microfibers in enzymatic hydrolysis. Imman et al. (2015) reported the destruction of the RS silica layers after alkaline-catalyzed liquid hot water pretreatment with a 0.25% NaOH solution. Mukherjee et al. (2018) found that NaOH pretreatment reduced the percentage of silica by favoring delignification due to silica links with lignin. Together with the efficient removal of lignin and silica, the low degradation of sugars and its non-corrosive nature make alkaline pretreatment one of the most suitable methods to use on RS (Vivek et al., 2019). In a previous study, RS delignification by NaOH pretreatment has provided adequate saccharification, recovering ~60% of reducing sugars from the original carbohydrates (Valles et al., 2021).

Integrated bioprocessing, in which multiple processing steps are combined in a single operation, is an attractive approach for industrial-scale butanol production, for which simultaneous saccharification and fermentation (SSF) and fermentation with *in situ* product recovery (ISPR) are two of the most promising strategies (Ibrahim et al., 2018). In SSF, hydrolysis and fermentation take place together in the same vessel. This process could avoid the glucose inhibition of hydrolytic enzymes because sugars are simultaneously released and consumed by the bacteria. Also, SSF of microwave-pretreated RS has been shown to be more efficient than separate hydrolysis and fermentation (SHF) in terms of butanol production and productivity (Valles et al., 2020). ISPR reduces the high cost of downstream recovery processing, which is one of the major challenges in the commercialization of biobutanol (Abo et al., 2019). At the same time, it improves fermentation performance by alleviating butanol inhibition, which occurs at concentrations > 10 g L^{-1} (Ahlawat et al., 2019;

Rochón et al., 2017). ISPR techniques include pervaporation, liquid extraction, gas stripping and perstraction. Of these, gas stripping is one of the simplest and most economic processes since it does not require either a membrane or chemicals and does not harm the culture (Li et al., 2020). By combining SSF with ISPR, the advanced SSFR (simultaneous saccharification, fermentation and recovery) configuration could markedly reduce the capital and operational costs of producing butanol from lignocellulosic biomass and food waste (Qureshi et al., 2020). Qureshi et al. (2006) carried out SSFR with *C. acetobutylicum* P260 from corn fiber arabinoxylan using gas stripping and reported that the full utilization of sugar and acids in SSFR, compared to SSF, increased ABE production (from 9.60 to 24.67 g L^{-1}) and productivity (from 0.20 to 0.47 g L^{-1} h^{-1}).

Combining SSFR with a fed-batch strategy, which allows large solid loadings without substrate or product inhibition is another method of drastically improving the cost-effectiveness of biobutanol production. Up to now, fed-batch has been considered to enhance ABE fermentation in SHF configurations (López-Linares et al., 2021; Rochón et al., 2017; Wen et al., 2018), but not in SSF, although fed-batch SSF was recently suggested as a promising alternative to be explored (Ibrahim et al., 2018). Feeding sterile substrate into the reactor is still a technical challenge to be overcome, although several studies on ethanol production by *Saccharomyces cerevisiae* have demonstrated the feasibility of feeding a sequential biomass to the reactor (Shengdong et al., 2006; Wang et al., 2013). For example, Shengdong et al. (2006) carried out fed-batch SSF in which an extra 10% (w/v) of pretreated RS was aseptically added in addition to a 10% (w/v) initial substrate concentration. This increased the reaction time by 144 h and achieved a much higher ethanol concentration (57.3 g L^{-1}) than that obtained in the single batch process (29.1 g L^{-1}). Fed-batch SSFR could thus solve some of the major challenges in ABE fermentation.

In the present work, a novel ABE fermentation approach was evaluated that consisted of fed-batch SSF with ISPR by gas stripping for butanol production from alkaline-pretreated RS. First, the effect of solid and enzyme loading on production was assessed in a batch SSF configuration using a central composite design (CCD). Based on the optimal values of solid and enzyme loading, fed-batch SSFR was then conducted with or without an additional enzyme dosage or medium compounds (buffer, yeast extract and minerals) in the subsequent feed cycles to further improve the economic viability of the process.

8.2. Materials and methods

8.2.1. Materials

RS from the Albufera Natural Park (Spain) was milled. The size fraction ranged from 100 to 500 μm was dried at 45 °C and stored. Its chemical composition (dry weight) was: glucan 35.6 ± 0.6%, xylan 17.6 ± 0.5%, arabinan 2.1 ± 0.2%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 10.4 ± 0.7%, ash 12.3 ± 0.7% and extractives 13.0 ± 1.3%. The Cellic® CTec2 commercial enzyme blend (Novozyme, Denmark) was used for enzymatic hydrolysis. A cellulase activity of 193 filter paper units (FPU) mL^{-1} was determined following the National Renewable Energy Laboratory (NREL) method (Adney and Baker, 1996) and additional information of the commercial enzyme blend can be found elsewhere (Aramrueang et al., 2017; dos Reis et al., 2013; Yang et al., 2017). *Clostridium beijerinckii* DSM 6422 (NRRL B-592) was purchased from DSMZ (Germany) and stored at -80 °C in a Reinforced Clostridial Medium (RCM) with 20% (v/v) glycerol. The pre-culture was statically grown for 24 h in 19 g L^{-1} RCM with 10 g L^{-1} glucose.

8.2.2. RS pretreatment

Alkaline pretreatment was conducted according to the protocol and the optimal conditions derived from a previous study (Valles et al., 2021). In brief, a solid loading of 5% (w/v) of RS was mixed with 0.75% (w/v) of NaOH solution and heated at 134 °C for 20 min. The solid fraction was then separated by centrifugation at 4000 rpm for 6 min (Mega Star 3.0, VWR, Germany) and washed several times with deionized water with a final pH adjustment to 6.5. The pretreated RS, which was previously dried at 45 °C for 48 h, was stored at -20 °C. After pretreatment a solid recovery of 48.19 ± 1.97% was obtained. The chemical composition of the alkaline-pretreated RS (dry weight) was: glucan 51.9 ± 0.6%, xylan 21.6 ± 0.3%, arabinan 3.8 ± 0.1%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 9.2 ± 0.5% and ash 7.4 ± 0.3%, indicating enriched carbohydrates with the removal of 57.5% acid insoluble lignin and 71.1% ashes.

8.2.3. Batch SSF

The SSF process was first optimized in 50-mL serum bottles in which solid (3.8 – 12.2% w/v) and enzyme loading (3.7 – 26.3 FPU g-dw^{-1}) were tested following the experimental design shown in Section 8.2.6. The medium (40 mL) contained: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} $C_2H_7NO_2$, 4 g L^{-1} of yeast extract, 0.09 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.001 g L^{-1} $MnSO_4{\cdot}H2O$ and 0.02 g L^{-1} $FeSO_4{\cdot}7H_2O$. After oxygen

displacement, the sealed bottles were autoclaved (121 °C for 10 min). Minerals (filter-sterilized by 0.22 µm) and enzyme were added before inoculation with 5% (v/v) of pre-culture. Incubation was conducted at 37 °C and 150 rpm for 72 h in an orbital shaker (SI500 model, Stuart, UK). Saccharification control without bacterial cells was conducted in triplicate with a solid loading of 8% (w/v) and an enzyme loading of 15 FPU g-dw^{-1}.

Once solid and enzyme loading were optimized, the process was scaled up in a 2-L stirred tank reactor (STR) using 500 mL of the above-mentioned medium with an RS loading of 9.2% (w/v). Start-up was similar to that performed with the serum bottles, but nitrogen was sparged after sterilization. After adding 4.73 mL of the enzyme blend (19.9 FPU g-dw^{-1}), the reactor was inoculated (5% v/v) and fermentation lasted 72 h at 37 °C and 120 rpm.

8.2.4. Fed-batch SSF coupled with *in-situ* gas stripping

The fed-batch SSF with ISPR by gas stripping took place in the 2-L STR. Figure 8.1 shows a schematic diagram of the integrated reactor setup. Gas stripping was performed by intermittently bubbling the fermentation gas (CO_2 and H_2) through the fermentation broth at 4 L min^{-1} by a vacuum gas pump (VP 86, VWR, Germany). The stripped solvents were recovered in a condenser at 4 °C with a cooling system (AD15R-30, VWR, USA). O_2-free distilled water was pumped by a peristaltic pump to keep constant the reactor volume at 500 mL. The condensate was periodically transferred for volume measurement and solvent analysis. The same start-up and operational conditions (37 °C and 120 rpm) described in Section 8.2.3 were used, ensuring an anaerobic environment by flushing the reactor headspace with nitrogen during feeding. Gas stripping started after 20 h and ended at 50 h. Dry alkaline-pretreated RS was added in three portions: 50% of the total quantity at the beginning of fermentation, 25% after 20 h and 25% after 30 h, thus doubling the solid loading from 9.2 to 18.4% (w/v). Three experiments were planned with different enzyme dosage and medium components together with the solid additions. In run 1, the enzyme blend Cellic® CTec2 was added to keep the enzyme loading constant at 19.9 FPU g-dw^{-1} throughout the entire process, while in runs 2 and 3 no extra enzyme was added to assess the Cellic® CTec2 cellulase activity over time. In runs 1 and 2, the fermentation was reinforced with medium components to keep the same ratio of buffer, yeast extract and minerals with feed solids, while in run 3 no additional medium components were used in order to assess the potential nutrient recycling from dead *C. beijerinckii* cells.

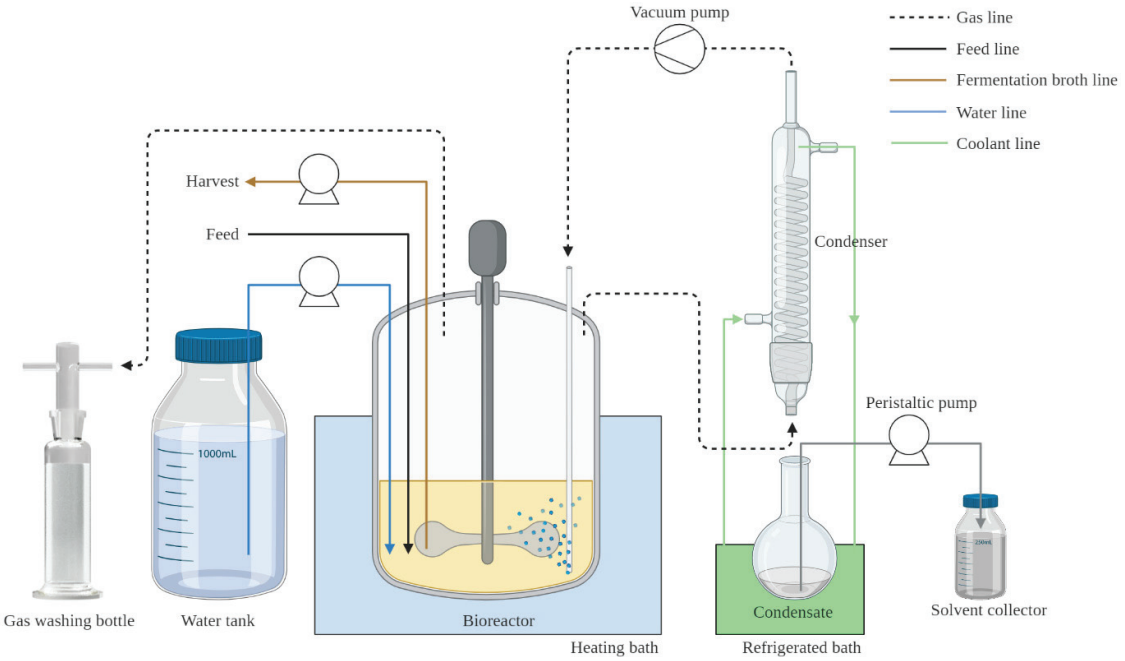

Figure 8.1. Schematic diagram of fed-batch SSF with *in-situ* product recovery by gas stripping (SSFR). Created with BioRender.com.

8.2.5. Analytical methods

The raw and pretreated RS were analyzed to determine the chemical composition according to NREL protocols (Sluiter et al., 2008). For all ABE fermentations, samples of 1 – 2 mL were periodically taken from the broth and, afterwards, were centrifuged (10000 rpm for 5 min) and filtered by 0.22-µm. A Minitrode electrode (Hamilton, USA) was used to pH measurements. The concentration of sugars (arabinose, glucose and xylose), inhibitory compounds (furfural, 5-HMF and levulinic acid) and fermentation products (acetone, butanol, ethanol, acetic acid and butyric acid) was determined by an Agilent HPLC (1100 Series, Agilent Technologies, USA) equipped with a refractive index detector, a diode array detector and an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA). The mobile phase (5 mM H_2SO_4) was set at 0.6 mL min^{-1}. The total phenolic compounds, expressed as gallic acid equivalents, was measured by the Folin-Denis method (Folin and Denis, 1912). A scanning electron microscope (SEM) S-4800 (Hitachi, Japan) was used to observe the *C. beijerinckii* DSM 6422 cells absorbed on the surface of the pretreated RS. Samples were coated with a gold and palladium mixture prior to imaging under SEM at an accelerating voltage of 20 kV.

8.2.6. Statistical design of experiments

Batch SSF was optimized by a CCD-based response surface method with concentration of butanol produced at 24 h (g L^{-1}) as the response variable in a total of 13 experiments with 5 central point replications. Solid loading (from 3.8 to 12.2% w/v) and enzyme loading (from 3.7 to 26.3 FPU g-dw^{-1}) were evaluated as the independent variables and their coded and real values are showed in Table 8.1. The statistical analysis was done on MINITAB® 19 software (Minitab Inc., USA). The optimal levels of the solid and enzyme loading predicted by the mathematical model were validated in triplicate. To obtain the product yield, a saccharification control was carried out in triplicate with a solid loading of 9.2% (w/v) and an enzyme loading of 19.9 FPU g-dw^{-1}.

Table 8.1. 5-Level CCD of 2 independent variables. α = 1.4142.

	Independent variables	Coded and real values				
		Level -α	Level -1	Central point (0)	Level +1	Level +α
X_1	Solid loading (% w/v)	3.8	5.0	8.0	11.0	12.2
X_2	Enzyme loading (FPU g-dw^{-1})	3.7	7.0	15.0	23.0	26.3

8.3. Results and discussion

8.3.1. Batch SSF: optimization

The optimum values of the solid and enzyme loading to maximize butanol production were assessed in batch SSF. For this, a five-level CCD was carried out using 50-mL serum bottles. The CCD experimental matrix with the real values of both independent variables is summarized in Table 8.2, along with the butanol production and sugar concentration in the culture broth at 24 h. The butanol titer at the end of fermentation (72 h) is also given along with the percentage of consumed sugars. These percentages were determined from the potential final sugar concentration estimated for each solid loading based on the sugar released by saccharification control (without inoculation).

At 24 h of fermentation, the butanol concentration ranged from 6.13 to 10.07 g L^{-1}, obtaining the minimum value from the lowest solid loading (3.8% w/v, run 5) and the maximum value from the solid loading of 11.0% (w/v, run 4). The central point replicates (run 9 – 13) show the low experimental variability of the parameters studied (butanol production: 9.95 ± 0.35 g L^{-1}; residual glucose: 1.33 ± 1.03 g L^{-1}, xylose: 5.02 ± 0.18 g L^{-1} and arabinose: 1.04 ± 0.05 g L^{-1}). After 72 h of fermentation, the maximum butanol concentration of 12.06 g L^{-1} was obtained with 8.0% (w/v, run

8), whereas higher solid loadings had a negative effect on the sugars converted to butanol with the consumption of reducing sugars below 70%. The negative impact of high biomass loading on ABE-SSF due to mass transfer limitations or the accumulation of inert components such as ashes, among other factors, was previously reported (Guan et al., 2016; Razali et al., 2018). In this work, the hydrolysis process seems not adversely impact by high solid loadings, due to the fact that a noticeable accumulation of glucose was observed for run 4 (16.73 g L^{-1}) and run 6 (25.58 g L^{-1}); both experiments corresponding to the combination of high solid loadings (≥11%) and high enzyme loadings (≥15 FPU g-dw^{-1}). Meanwhile, low acid concentrations were observed at 24 h for the whole set of experiments (acetic acid ranging from 0.95 to 1.38 g L^{-1}, butyric acid ranging from 0.60 to 1.89 g L^{-1}, total free acid concentration less than 6 mM) which were accompanied by butanol concentrations higher than 6 g L^{-1}. Thus, indicating a quick transition from acidogenesis to solventogenesis metabolism, being acidogenesis the limitation step at high solid loadings. These results corroborated the importance of the solid loading for an efficient biomass processing on simultaneous saccharification and fermentation.

Table 8.2. CCD experimental matrix along with the values of butanol production (g L^{-1}) at 24 and 72 h, remaining sugars (g L^{-1}, glucose, xylose and arabinose) at 24 h and consumed sugars (%, glucose and xylose) at 72 h.

Run	Real values[a]		24 h				72 h		
			Buta-nol	Sugars (g L^{-1})			Buta-nol	Consumed sugars (%)[b]	
	X_1	X_2	(g L^{-1})	Glucose	Xylose	Arabinose	(g L^{-1})	Glucose	Xylose
1	5.0	7.0	6.70	0.25	1.92	0.33	8.49	96.4	85.6
2	11.0	7.0	9.34	4.17	4.47	1.00	10.56	59.6	63.7
3	5.0	23.0	7.64	0.38	1.96	0.14	8.35	97.2	75.9
4	11.0	23.0	10.07	16.73	5.77	1.41	11.74	68.2	68.7
5	3.8	15.0	6.13	0.29	1.08	0.00	6.61	96.9	84.4
6	12.2	15.0	8.23	25.58	7.09	1.44	10.37	50.4	62.7
7	8.0	3.7	7.74	0.19	2.96	0.38	8.86	76.0	72.7
8	8.0	26.3	9.88	6.00	4.43	0.99	12.06	88.8	72.7
9-13	8.0	15.0	9.95 ± 0.35	1.33 ± 1.03	5.02 ± 0.18	1.04 ± 0.05	10.87 ± 0.46	94.2 ± 0.6	60.6 ± 2.8

[a] X_1: solid loading (% w/v); X_2: enzyme loading (FPU g-dw^{-1})

[b] Final sugar concentration of saccharification control (solid loading of 8% w/v and an enzyme loading of 15 FPU g-dw^{-1}): 39.95 ± 0.23 g L^{-1} glucose, 13.35 ± 0.16 g L^{-1} xylose and 1.86 ± 0.00 g L^{-1} arabinose

Regarding the effect of enzyme dosing, increasing enzyme loading from 7.0 (run 1) to 23.0 FPU g-dw^{-1} (run 3) led to an improvement of 14% in butanol production at 24 h (from 6.70 to 7.64 g L^{-1}) when 5.0% (w/v) RS was used.

Nevertheless, with the same degree of enzyme increase (7.0 to 23.0 FPU g-dw^{-1}) but with 11.0% (w/v) RS, 24-h butanol production slightly improved from 9.34 (run 2) to 10.07 g L^{-1} (run 4). At the end of the fermentation, butanol concentration in run 4 reached 11.74 g L^{-1} with around 68% of the glucose and xylose consumed, so that the combination of high solid and enzyme loading did not achieve the best use of the biomass. 10 g L^{-1} is most likely a sub-lethal concentration of butanol for *C. beijerinckii* DSM 6422 that slows down the consumption of the sugar released when a high enzyme loading is used. Although it was not observed in these experiments, several authors have found that large amounts of enzyme in SSF processes can reduce butanol production and productivity by sugar inhibition and cellulase stress (Dong et al., 2016; Razali et al., 2018). Interestingly, ~9 g L^{-1} butanol was produced at 72 h from 8.0% (w/v) of pretreated RS with only 3.7 FPU g-dw^{-1} (run 7), showing alkaline-pretreated RS could be saccharified with low enzyme consumption. The results obtained from 8.0% (w/v) of solid showed that the use of 15.0 FPU g-dw^{-1} (run 9 – 13) is enough to achieve the same butanol production (1% of difference) at 24 h when almost twice the amount of enzyme is used (26.3 FPU g-dw^{-1}, run 8). The differences between both enzyme doses at the end of the fermentation shows a 10% variation in butanol production. This evidence is extremely important since the economic viability of the process is guaranteed by not adding extensive amounts of enzyme.

The results of the integrated SSF (Table 8.2) show that at least 79% of the final butanol production was reached at 24 h, thus indicating the fast fermentation profile obtained. With the aim of maximizing butanol production prior to developing the fed-batch SSFR alternative, instead of 72 h, butanol production at 24 h was therefore selected as the response variable in the optimization. After fitting the experimental data by means of a linear regression analysis, the following second-order model was obtained:

$$Butanol\,production(24h) = -4.77 + 2.630X_1 + 0.307X_2 - 0.1413X_1^2 - 0.00718X_2^2 - 0.0023X_1X_2 \quad (1)$$

where X_1 is the solid loading (% w/v) and X_2 is the enzyme loading (FPU g-dw^{-1}). Table 8.3 shows the analysis of variance (ANOVA) and the coded regression coefficients of the above quadratic model. At a confidence level of 95%, while the model was significant (p-value = 0.0006), as the lack-of-fit was not significant (p-value = 0.1466), Equation 8.1 could accurately predict the effect of solid and enzyme loading on butanol production at 24 h. The good agreement between the observed and predicted data was indicated by the high values of the coefficient of determination (R^2: 0.9315) and the adjusted coefficient of determination (Adj. R^2:

0.8826). According to R^2, only ~7% of the total disparity was not explained by the model. Furthermore, a standard deviation of 0.4887 g L^{-1} denoted the small difference between the experimental and fitted data in terms of units of the response. As can be seen in Table 8.3, for both the solid (X_1) and the enzyme (X_2), the p-values of linear (X_1 = 0.0006, X_2 = 0.0113) and quadratic effects (X_1X_1 = 0.0002, X_2X_2 = 0.0422) were lower than 0.0500, so that all the effects of the two factors evaluated were found to be significant. No interaction was found between solid and enzyme loading (X_1X_2, p-value = 0.8310). The relative importance of the variables, based on the coded coefficients, was as follows: solid loading (X_1 = 1.01, X_1X_1 = -1.27) > enzyme loading (X_2 = 0.59, X_2X_2 = -0.46).

Table 8.3. ANOVA of the CCD model for butanol production (g L^{-1}) at 24 h.

Source	Degrees of freedom	Sum of squares	Mean square	F value	p-value Prob > F	Coeffi-cient[a]
Model	5	22.74	4.55	19.04	0.0006	
Linear	2	10.87	5.44	22.76	0.0009	
X_1: solid loading (% w/v)	1	8.10	8.10	33.90	0.0006	1.01
X_2: enzyme loading (FPU g-dw^{-1})	1	2.77	2.77	11.61	0.0113	0.59
Square	2	11.86	5.93	24.82	0.0007	
X_1X_1	1	11.25	11.25	47.09	0.0002	-1.27
X_2X_2	1	1.47	1.47	6.15	0.0422	-0.46
2-way interactions	1	0.01	0.01	0.05	0.8310	
X_1X_2	1	0.01	0.01	0.05	0.8310	-0.05
Error	7	1.67	0.24			
Lack-of-fit	3	1.18	0.39	3.18	0.1466	
Pure error	4	0.49	0.12			
Total	12	24.41				
Standard Deviation, SD					0.4887	
R^2					0.9315	
Adj. R^2					0.8826	

[a] For coded variables

The 3D response surface plot derived from the regression model and the corresponding 2D contour plot are shown in Figure 8.2. According to the ANOVA results, these plots indicate that the variation of solids has a greater impact than that of the enzyme on the response and show the non-interaction between both factors. The maximum butanol production of 10.32 g L^{-1} at 24 h from 9.2% (w/v) solid and 19.9 FPU g-dw^{-1} enzyme loadings was estimated by the model. Butanol concentration rises to a peak value by increasing solid loading to 9.2% (w/v), since more fermentable sugars are released. From 9.2% to 12.2% RS (w/v), the response

decreases because the metabolism of *C. beijerinckii* DSM 6422 seems to be adversely impacted by the buffering effect of some RS compounds such as ash. Regarding enzyme loading, Figure 8.2 shows a flat area (~13 to 26 FPU g-dw^{-1}) around the optimum value in which varying enzyme loading would reduce butanol production by less than 3%, so that the model confirmed that neither sugar inhibition nor cellulase stress occurred.

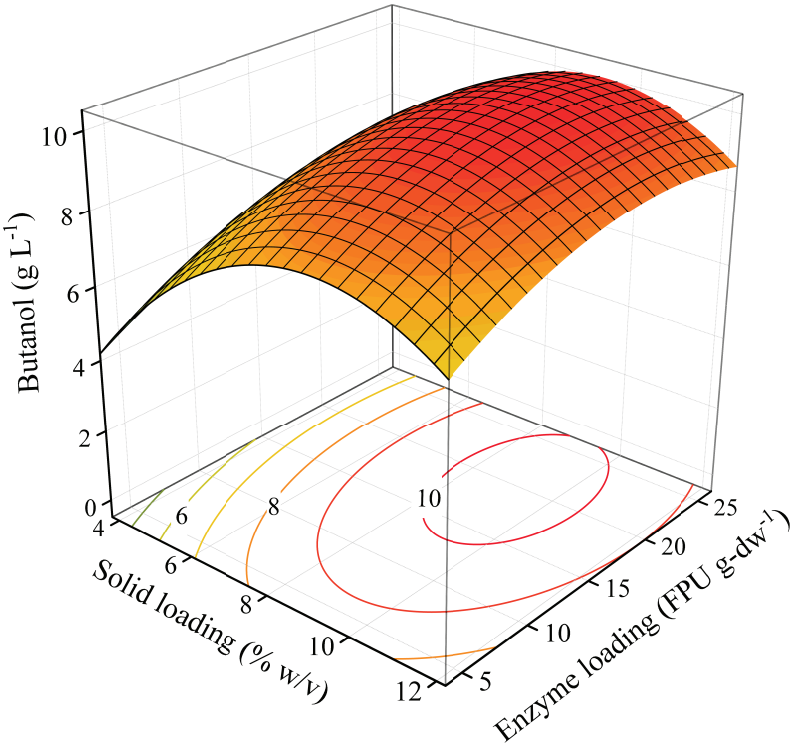

Figure 8.2. Response surface and corresponding contour plot for butanol production (g L^{-1}) at 24 h: combined effect of solid loading (% w/v) and enzyme loading (FPU g-dw^{-1}).

8.3.2. Batch SSF: model validation

The butanol production model at 24 h predicted by the CCD was validated through three identical assays performed on 50-mL serum bottles. Based on the optimal settings, 9.2% (w/v) alkaline-pretreated RS was hydrolyzed and fermented simultaneously by 19.9 FPU g-dw^{-1} of enzyme. Figure 8.3 a depicts the time fermentation profile of the products (acetone, butanol, acetic acid and butyric acid) and sugars (glucose, xylose and arabinose). Ethanol was not detected as it has been previously reported by others authors using strain *Clostridium beijerinckii* DSM 6422 (Plaza et al., 2017; Valles et al., 2021, 2020). At 12 h, before *Clostridium* metabolism was entirely active, 23.78 ± 4.67 g L^{-1} of sugars (17.74 ± 3.50 g L^{-1} glucose, 5.19 ± 1.02 g L^{-1} xylose and 0.85 ± 0.16 g L^{-1} arabinose) remained in the medium. From the subsequent uptake of monosaccharides, 9.27 ± 0.87 g L^{-1} of butanol and 15.52 ± 1.19

g L^{-1} of ABE (butanol:acetone mass ratio = 1.48) were produced at 24 h. A slightly lower production (~10%) was obtained by comparing the model's predicted response (10.32 g L^{-1}). This small discrepancy was due to a minor delay in fermentation, as it only takes 12 h to reach 10.67 g L^{-1} (Figure 8.3 a). When fermentation finished at 72 h, 11.89 ± 0.49 g L^{-1} of butanol and 19.42 ± 1.46 g L^{-1} of ABE (butanol:acetone mass ratio = 1.58) were obtained, thus giving a butanol productivity of 0.165 ± 0.007 g L^{-1} h^{-1} and an ABE productivity of 0.270 ± 0.020 g L^{-1} h^{-1}. The butanol productivity increases to 0.386 ± 0.036 g L^{-1} h^{-1} when considering 24 h, in which time 78% of the butanol had already been produced. The butanol-biomass ratio as parameter to assess the mass balance of the whole process from raw RS to butanol was calculated to be 62.6 ± 2.6 g-butanol kg-raw RS^{-1}. In the control assay conducted under optimal conditions but without inoculation, sugar concentration reached 60.00 ± 5.93 g L^{-1} (44.50 ± 2.19 g L^{-1} glucose, 13.36 ± 3.55 g L^{-1} xylose and 2.14 ± 0.19 g L^{-1} arabinose) at 72 h. From these values, butanol and ABE yields of 0.253 ± 0.020 and 0.412 ± 0.020 g g of consumed $sugar^{-1}$ were obtained. In comparison with those in the literature, the final butanol concentration was within the greatest values (10.2 – 13.0 g L^{-1}) reported for SSF systems from cellulosic material and different species of *Clostridium* (Dong et al., 2016; Guan et al., 2016; Qi et al., 2019). Whereas higher values of butanol-biomass ratio (80 – 110 g-butanol kg-raw RS^{-1}) have been reported for other cellulosic materials fermented in SSF systems (Guan et al., 2016; Qi et al., 2019; Razali et al., 2018), butanol-biomass ratio obtained in this study, 62.6 ± 2.6 g-butanol kg-raw RS^{-1}, improves the rice straw conversion to butanol in 23% from SSF process (Valles et al., 2020). Results are comparable not only in terms of butanol production but also in productivity to Dong et al. (2016), who achieved 13.0 g L^{-1} of butanol in 48 h using 9% (w/v) of alkaline-pretreated corn stover and *C. saccharobutylicum* DSM 13864. In contrast, SSF with *C. acetobutylicum* ATCC 824 (Guan et al., 2016; Qi et al., 2019) showed a slower fermentation rate and low butanol production at 24 h (less than 4 g L^{-1}) and requiring between 120 and 144 h to obtain the above-mentioned final concentrations. *C. beijerinckii* DSM 6422 therefore seems to be a good candidate for SSF together with *C. saccharobutylicum* DSM 13864. In comparison with the previous results of *C. beijerinckii* DSM 6422 on SHF of hydrolyzates from 8% (w/v) of alkaline-pretreated RS (Valles et al., 2021), the overall butanol productivity (considering both hydrolysis and fermentation time) was 2.4 times higher in the one-step (SSF, 0.165 g L^{-1} h^{-1}) than two-step process (SHF, 0.070 g L^{-1} h^{-1}). This confirms that one of the SSF's great advantages over SHF is the time savings, which reduces operational costs, along with a reduced risk of glucose contamination and enzyme inhibition.

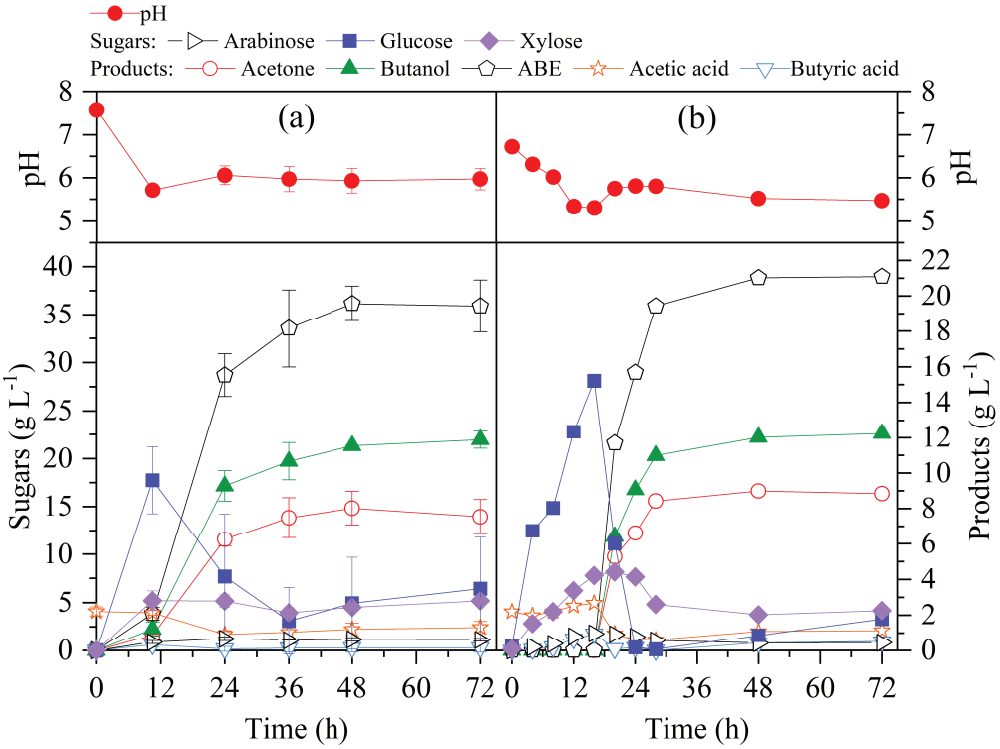

Figure 8.3. CCD model validation. (a) Batch SSF in a 50-mL serum bottles. (b) Scale-up of the batch SSF to a 2-L reactor.

The optimized batch SSF was carried out in a 2-L STR with a working volume of 500 mL to evaluate the feasibility of the process in a bench-scale bioreactor. Scale-up was successful as no relevant differences were found between the sugar and product profiles of both experiments (Figure 8.3). The butanol concentration at 24 h (9.06 g L^{-1}) and 72 h (12.24 g L^{-1}) in the 2-L reactor differed by less than 3% with respect to the values observed in the serum bottles, corresponding to a final conversion of raw RS to butanol of 64.4 g-butanol kg-raw RS^{-1}. The final production of ABE was 8% higher (21.09 g L^{-1}, butanol:acetone mass ratio = 1.38) and the productivity was 0.170 g L^{-1} h^{-1} for butanol and 0.293 g L^{-1} h^{-1} for ABE. At 72 h, 8.27 g L^{-1} of sugars (3.29 g L^{-1} glucose, 4.17 g L^{-1} xylose and 0.81 g L^{-1} arabinose) remained unused in the medium. Taking into account the sugar released in the control experiments without inoculation, 86% of the sugars were consumed, thus resulting in butanol and ABE yields of 0.237 and 0.408 g g of consumed $sugar^{-1}$, respectively. The maximum concentrations of total phenolic compounds (0.42 – 0.45 g L^{-1}) were just about half the inhibitory concentration (0.71 g L^{-1}) for *C. beijerinckii* DSM 6422 (López-Linares et al., 2019). Furfural, 5-HMF and levulinic acid were not detected and the maximum concentration of undissociated acids (acetic and butyric) was 12.51 mM, below the inhibitory level (16 mM, Valles et al., 2021). The end of butanol production and the observed increase in the concentration of residual sugars from 48 to 72 h thus suggests that fermentation was inhibited by butanol at a

concentration of 12.24 g L^{-1}. Ahlawat et al. (2019) found 12.56 g L^{-1} as the threshold concentration of butanol, at which the growth of *C. acetobutylicum* MTCC 11,274 and sugar consumption stopped. The inhibitory concentration for *C. acetobutylicum* DSM 792 was 10.5 g L^{-1} (Rochón et al., 2017), showing that tolerance depends, among other factors, on the bacteria strain. To further improve RS butanol production and productivity, the optimized operational conditions (9.2% (w/v) of solid and 19.9 FPU g-dw^{-1} of enzyme loadings) were selected as the initial conditions of the fed-batch SSFR configuration.

8.3.3. Fed-batch SSF coupled with *in-situ* gas stripping

A novel fed-batch SSF with ISPR by gas stripping was evaluated to assess the feasibility of using high amounts of biomass in ABE fermentation while avoiding both substrate and butanol inhibition. From an initial alkaline-pretreated RS loading of 9.2% (w/v), two additional biomass feedings were performed (as indicated by the arrows in Figure 8.4) to avoid inefficient mixing or low water activity associated with the high amount of solids to be processed (18.4% w/v). The first additional feeding (25% of the whole) was done at 20 h, before expecting complete glucose depletion (Figure 8.3 b). This model helps to maintain biological activity once the solventogenesis has started. The second (25% of the whole) was carried out 10 h after the first, when solubilization of the previously added solid was ensured. Gas stripping was turned on at 20 h with the first biomass feeding to avoid butanol inhibition. The application time of gas striping was planned to keep the butanol concentration in the fermentation medium well below 12 g L^{-1}. It was turned off at 50 h when the concentration was less than 5 g L^{-1}. Three experimental runs were carried out to assess the recycling of enzyme and medium compounds (buffer, yeast extract and minerals) in the process.

The fermentation profile of run 1, where the final enzyme loading (19.9 FPU g-dw^{-1}) and the nutrient ratio over solids were the same as in the batch SSF, is depicted in Figure 8.4 a. As shown, the pH and sugar profile in the first 20 h was very similar to those of the batch SSF (Figure 8.3 b). The pH then remained stable at 5.74 ± 0.17 until the end of fermentation due to the re-assimilation of acids into solvents, with low concentrations of acetic acid (0.76 – 2.98 g L^{-1}) and butyric acid (0.00 – 0.40 g L^{-1}) since 20 h. The maximum observed concentration of undissociated acids (10.92 mM) was below the inhibitory value (16 mM). Regarding other potential inhibitors, only total phenolic compounds were found at the end of the process, but at a non-inhibitory concentration (0.70 g L^{-1}). The fed-batch strategy allowed processing in 72 h a total RS loading of 18.4% (w/v), which is equivalent to a gradual feeding of 120 g L^{-1} of sugars (89.00 g L^{-1} glucose, 26.72 g L^{-1} xylose and 4.28 g L^{-1} arabinose)

according to the saccharification control where 60 g L^{-1} of sugars were obtained when half of the solid loading was used. As fed-batch SSF successfully avoided product inhibition of saccharification, with this approach the low sugar yield typically found in SHF when high sugar concentrations are processed (Zhu et al., 2005) is completely eliminated. While glucose accumulation stopped after 25 h, the xylose concentration remained at 5.59 ± 1.77 g L^{-1} from 25 h to 72 h because a balance was established between hydrolytic enzyme activity and bacterial metabolism. *C. beijerinckii* DSM 6422 consumed 97% of the released sugars (100% of glucose and arabinose and 85% of xylose). After analyzing the condensates from gas stripping, a total volume of 150 mL was recovered at the end of the process with an average butanol concentration of 32.21 ± 3.91 g L^{-1} and ABE of 45.14 ± 4.52 g L^{-1}. The cumulative concentrations of solvents were calculated by dividing the mass of the solvents in the reactor plus those recovered in the condenser by the reactor volume. Cumulative ABE production at 72 h was thus 38.36 g L^{-1} (butanol:acetone mass ratio = 1.73), giving an ABE productivity of 0.533 g L^{-1} h^{-1}. Of the ABE solvents, 24.33 g L^{-1} were butanol, resulting in a butanol productivity of 0.338 g L^{-1} h^{-1} lower than the average butanol stripping rate (0.562 g L^{-1} h^{-1}) enabling the decrease on the butanol concentration in the reactor. The butanol selectivity, defined elsewhere (Qureshi et al., 1992), was calculated to 4.96 ± 0.97. In comparison with butanol selectivity (between 4 and 22.57) reported by other gas stripping studies (Qureshi et al., 2014, 2006), the selectivity of this experiment is in the lower range, so that it could be improved decreasing the gas flow rate or the cooling temperature as it has been suggested by Xue et al. (2014). Compared with the butanol (12.24 g L^{-1}) and ABE (21.09 g L^{-1}) production in the batch SSF (Figure 8.3 b), the values obtained in run 1 nearly doubled, as did the productivity, as the fermentation time was the same (72 h), improving slightly the butanol-biomass ratio up to 64.0 g-butanol kg-raw RS^{-1}. Considering the results obtained in the system without product recovery, the butanol yield fell by 11% (from 0.237 to 0.210 g g of consumed $sugar^{-1}$) and that of ABE by 19% (from 0.408 to 0.330 g g of consumed $sugar^{-1}$). The moderate reduction in butanol and ABE yield could be associated with the fact that the cells could increase their maintenance energy expenditure when they are continuously exposed to a sub-lethal butanol concentration (Branska et al., 2018). SEM images showed a high amount of long-chain *C. beijerinckii* DSM 6422 cells adhered to the altered surface of the RS at 20 h. The micro-fibrous cellulose structures were used as an immobilization carrier, although no biofilm was found. Unlike other *Clostridium* strains such as *C. acetobutylicum*, *C. beijerinckii* does not form biofilms as it has a weak cell-to-cell communication system (Liu et al., 2018). At 50 h, SEM images showed a small number of cells on the biomass support and some were identified as mother cells for spores, since they showed a swollen clostridial shape.

The broth became viscous at this time, probably due to the autolysis promoted by sporulation as a natural survival strategy for the long-term (Branska et al., 2018). A third feeding of RS was added at 72 h but the fermentation did not progress further (data not shown). From SEM images it could be seen that the outer surface of the rice straw has been significantly affected by the enzymatic degradation after 72 h. Compared with the reported ABE-SSF studies, the final solid loading used (18.4% w/v) was much higher than those typically found in the literature, which range from 7.4 to 13% (Dong et al., 2016; Gallego et al., 2015; Guan et al., 2016; Qi et al., 2019; Razali et al., 2018). Only Li et al. (2016) were able to efficiently use a similar amount of lignocellulosic feedstock (17.5% w/w of steam-exploded corn straw) by means of an intensification method such as periodic peristalsis.

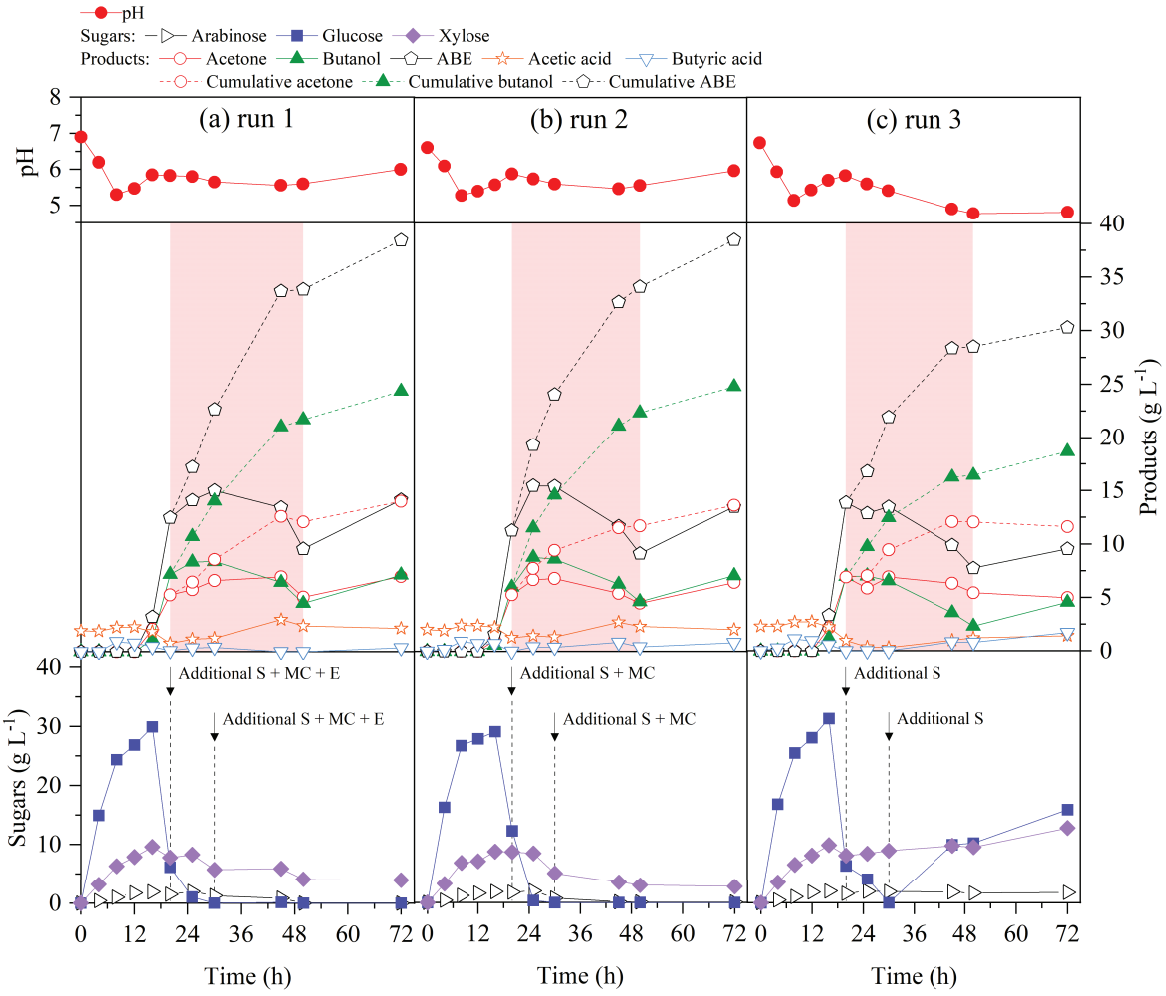

Figure 8.4. Time course of the fed-batch SSFR in a 2-L reactor. Additional solid (S) was added with medium compounds (MC) and enzyme (E) in run 1, with MC but without E in run 2 and without MC and E in run 3. Red shaded region indicates the application period of gas striping. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Once it had been shown that fed-batch SSFR could efficiently produce butanol from lignocellulosic waste, two additional experiments were planned with the aim of improving the process's economics (runs 2 and 3). In run 2 (Figure 8.4 b), the medium compounds ratio over solids was kept as in run 1, but no extra doses of enzyme were incorporated, which halved the final enzyme loading (9.95 FPU g-dw^{-1}). The same cumulative butanol (24.80 g L^{-1}) and ABE (38.47 g L^{-1}, butanol:acetone mass ratio = 1.81) production were attained by using half the enzyme dosing, giving a butanol productivity of 0.344 g L^{-1} h^{-1}, an ABE productivity of 0.534 g L^{-1} h^{-1} and a butanol-biomass ratio of 65.3 g-butanol kg-raw RS^{-1}. No remarkable differences were found in the profiles of pH, products and sugars between run 1 (Figure 8.4 a) and run 2 (Figure 8.4 b). These results suggest that the activity of cellulase, β-glucosidase and hemicellulose combined in the Cellic® CTec2 blend lasted for the whole fed-batch SSFR without further enzyme addition, which is a great economic saving. It is important to consider that, depending on the selected substrate, the hydrolytic enzyme can account for over 12% of operational costs in an integrated process such as SSFR (Qureshi et al., 2020). Simultaneously repeated hydrolysis and fermentation (SRHF) is another strategy for enzyme recycling, where sequential cycles of both stages are conducted in parallel in separate vessels (Zhao et al., 2019). Unlike cellulases and xylanases, β-glucosidase without a cellulose-binding module does not bound to lignocellulosic substrates (Várnai et al., 2011), so that β-glucosidase is gradually lost in SRHF, notably reducing the glucose concentration over the saccharification cycles (Zhao et al., 2019). One of the advantages of the process of this work is thus the use of a sole dosage of enzyme blend to hydrolyze sequential biomass additions. The fermentation profile in run 3, in which neither extra medium compounds nor enzymes were added after inoculation, is shown in Figure 8.4 c. While in previous experiments pH was recovered from 30 h (second biomass addition) to the end of fermentation, in this case pH decreased from 5.41 to 4.81, so that not adding ammonium acetate adversely impacts the buffering capacity, as this compound was used as a solvent precursor. Although under these pH conditions the undissociated acids did not reach inhibitory concentrations, 25% of the sugars released from the RS remained unused, showing that nutrients were limited. The incomplete uptake of sugars by bacterial cells led to a 20% reduction in the cumulative production of butanol (18.67 g L^{-1}), ABE (30.30 g L^{-1}, butanol:acetone mass ratio = 1.60) and butanol-biomass ratio (49.1 g-butanol kg-raw RS^{-1}) at 72 h compared to previous experiments. The results of this work confirm that the nitrogen, vitamins, amino acids and minerals supplied by the initial yeast extract dosage were not enough. In a previous study on SSF optimization from microwave-pretreated RS, the regression model predicted a 17% reduction in butanol

production by *C. beijerinckii* DSM 6422 (from 5.28 to 4.37 g L^{-1}) when yeast extract was halved from 4 to 2 g L^{-1} (Valles et al., 2020).

Results show that the fed-batch SSF configuration previously investigated to produce ethanol from biomass (Shengdong et al., 2006; Wang et al., 2013) is also a feasible configuration for butanol production from lignocellulosic waste. By reducing the overall process time by combining saccharification, fermentation and product recovery, high butanol productivities with one enzyme dosage were achieved. Indeed, higher productivities than the alternative configurations for improving butanol productivity such as fed-batch SHF or continuous fermenters were obtained. For example, fed-batch SHF with product recovery has been used to ferment sugarcane-sweet sorghum juices, obtaining a productivity of 0.13 g L^{-1} h^{-1} (Rochón et al., 2017), while suspended-growth cell continuous processes reported productivities ranging from 0.18 to 0.23 g L^{-1} h^{-1} with lignocellulosic waste (Al-Shorgani et al., 2019; Van Hecke and De Wever, 2017). Fed-batch SSFR is in fact a promising configuration for improving ABE productivity, as it avoids the strain degeneration problem of a continuous process and SHF substrate inhibition. However, further research is required to avoid autolysis and extend the operational time by implementing, for instance, a fermenter bleeding strategy.

8.4. Conclusions

An advanced configuration based on simultaneous saccharification and fermentation combined with gas stripping (SSFR) was shown to be a suitable configuration to produce butanol from lignocellulosic waste. SSFR operated in fed-batch quickly processed high solid loadings while avoiding both substrate and product inhibition, allowing butanol productivity as high as 0.344 g L^{-1} h^{-1} (titer of 24.80 g L^{-1}) by processing an RS loading of 18.4% (w/v) in the short operational time (hydrolysis + fermentation) of 72 h. The enzyme was efficiently used, as no additional enzyme dosing was necessary, thus improving the process's economic viability.

CRediT authorship contribution statement

Alejo Valles: methodology, investigation, formal analysis, writing-original draft, visualization. **Javier Álvarez-Hornos:** conceptualization, methodology, investigation, writing-review and editing, supervision, project administration. **Miguel Capilla:** methodology, visualization. **Pau San-Valero:** conceptualization, methodology. **Carmen Gabaldón:** conceptualization, methodology, investigation, writing-review and editing, supervision, project administration, funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

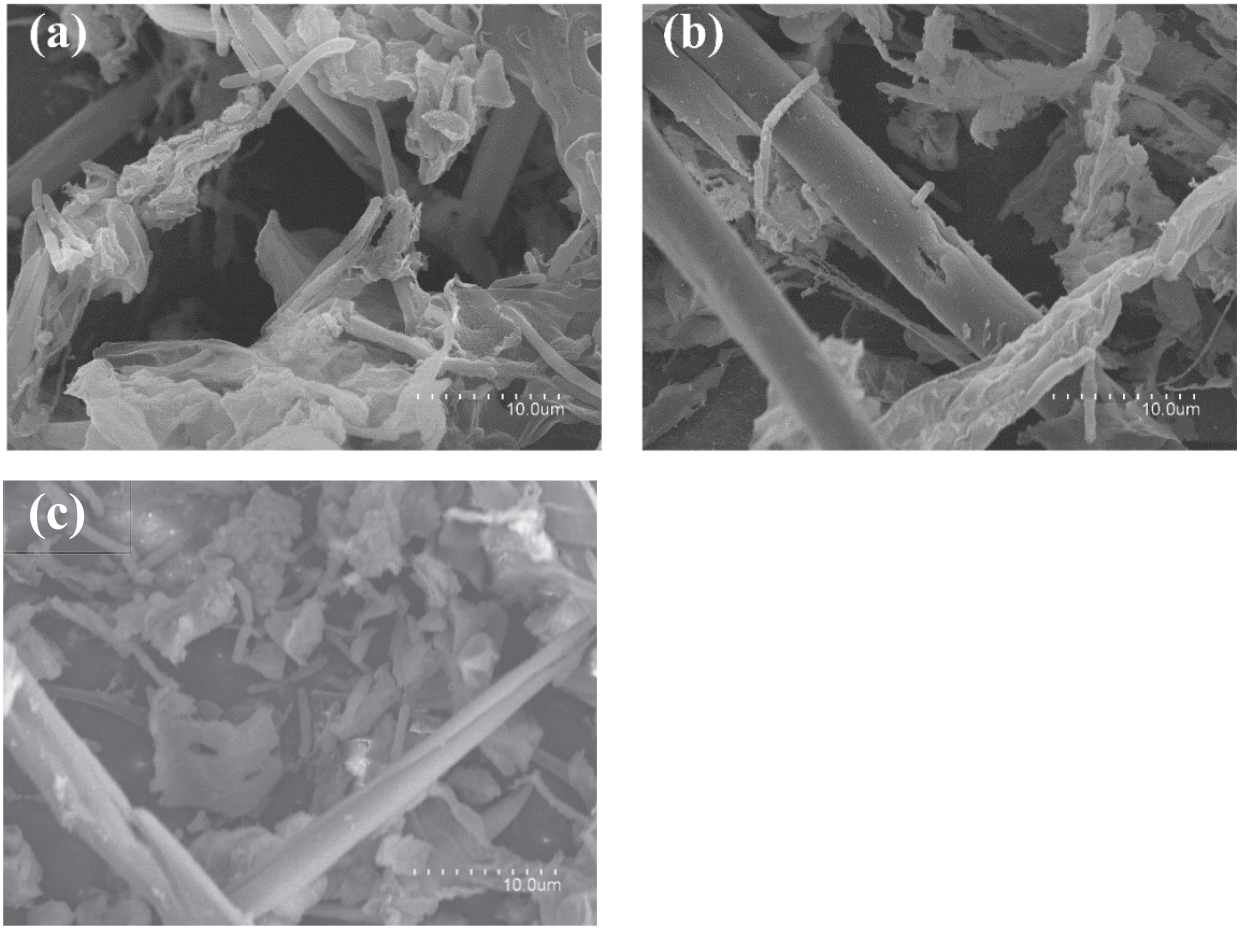

Figure 8.S1. SEM images of cells of *C. beijerinckii* DSM 6422 grown on the surface of alkaline-pretreated RS at 20 h (a), 50 h (b) and 72 h (c) of the fed-batch SSFR. The photographs have been taken at 3000 magnification.

Table 8.S1. CCD experimental matrix along with the values of pH and acids (acetic and butyric, g L^{-1}) at 24 and 72 h.

Run	Real values[a]		24 h			72 h		
			pH	Total acids (g L^{-1})		pH	Total acids (g L^{-1})	
	X_1	X_2		Acetic	Butyric		Acetic	Butyric
1	5.0	7.0	6.06	1.34	0.60	5.34	1.42	1.32
2	11.0	7.0	6.19	1.00	1.44	6.00	1.33	0.00
3	5.0	23.0	5.53	1.38	1.12	5.21	1.62	1.28
4	11.0	23.0	6.28	0.93	0.87	5.98	0.96	0.00
5	3.8	15.0	5.59	1.27	0.98	5.29	1.26	1.71
6	12.2	15.0	6.14	0.95	1.89	5.92	1.50	0.00
7	8.0	3.7	5.76	1.71	0.61	5.21	2.33	1.55
8	8.0	26.3	6.12	0.98	1.49	5.81	1.22	0.00
9-13	8.0	15.0	5.85 ± 0.23	1.21 ± 0.19	1.08 ± 0.26	5.46 ± 0.13	1.56 ± 0.23	0.64 ± 0.25

[a]X_1: solid loading (% w/v); X_2: enzyme loading (FPU g-dw^{-1})

8.5. References

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9. CONCLUSIONES Y PERSPECTIVAS

En esta tesis doctoral, se ha estudiado el proceso de sacarificación y fermentación simultánea (SSF) como alternativa al proceso convencional de sacarificación y fermentación separada (SHF) en la producción de biobutanol a partir de paja de arroz. Las principales conclusiones que se extraen de los resultados obtenidos se exponen a continuación:

I. Se ha demostrado que el proceso SSF, como estrategia que combina las etapas de hidrólisis enzimática y de fermentación, puede aplicarse en la producción de biobutanol a partir de paja de arroz, obteniéndose un incremento en la productividad de hasta el 173% en comparación con el proceso SHF.

II. Los resultados obtenidos en la aplicación del proceso SSF a paja de arroz pretratada con hidróxido sódico muestran una mayor producción (12.2 g/L) y una mayor productividad de butanol (0.170 g/L h) que aquellos obtenidos tras el pretratamiento de hidrotermólisis asistida por microondas, con unos valores máximos de producción y productividad de butanol de 5.5 g/L y 0.114 g/L h, respectivamente. Así mismo, este proceso operado en modo de alimentación semicontinuo y combinado con recuperación *in situ* de producto (SSFR) permite procesar en tan solo 72 horas el doble de la cantidad de biomasa (18.4% p/v) utilizada en el proceso en modo discontinuo, incrementándose la producción de butanol hasta 24.80 g/L y la productividad hasta 0.344 g/L h. Por lo tanto, se ha demostrado que el proceso SSFR en modo semicontinuo es una configuración avanzada que puede mejorar la eficiencia de la producción de biobutanol a partir de residuos lignocelulósicos.

III. El análisis de los efectos de los parámetros del proceso SSF sobre la producción de biobutanol muestra que la carga enzimática es un factor clave en el aprovechamiento de la paja de arroz pretratada tanto mediante hidrotermólisis asistida por microondas como con hidróxido sódico, con valores óptimos de 13.5 y 19.9 FPU/g, respectivamente. Otros factores con efecto significativo sobre la producción de biobutanol son la concentración de extracto de levadura con un valor óptimo de 4.7 g/L y la carga de sólidos con un valor óptimo de 9.2% (p/v).

IV. Es posible realizar un cambio de escala con un factor de ∼10 del proceso SSF utilizando las condiciones de operación optimizadas en los ensayos llevados a cabo en un volumen de 40 mL, ya que se ha observado que los perfiles de consumo de

monosacáridos y de producción de ácidos y disolventes son similares entre los ensayos de 40 y 500 mL.

V. Es posible reducir la carga enzimática a la mitad (de 19.9 a 10.0 FPU/g) en el proceso SSFR en modo semicontinuo sin afectar a la producción y a la productividad de biobutanol. No obstante, es necesario realizar dosis adicionales de compuestos tampón, extracto de levadura y minerales para evitar una disminución del 20% en la producción de biobutanol.

VI. Las condiciones de operación empleadas en la hidrotermólisis asistida por microondas (200 °C, 15 minutos y carga de sólidos del 10% p/v) no son las adecuadas para garantizar un cambio sustancial de la composición química de la paja de arroz, obteniéndose un reducido porcentaje de eliminación de lignina (13%). En este sentido, la lignina remanente en la biomasa afecta negativamente a la posterior etapa de hidrólisis enzimática, liberándose concentraciones de monosacáridos comprendidas entre 23.0 y 26.5 g/L. Los resultados muestran la necesidad de estudiar métodos de pretratamiento alternativos u otras condiciones de operación en el pretratamiento con microondas.

VII. El uso de concentraciones de hidróxido sódico comprendidas entre 0.5 y 2.0% (p/v) en el pretratamiento alcalino de la paja de arroz permite obtener mayores porcentajes de eliminación de lignina de la biomasa (58 – 98%) que los alcanzados en la hidrotermólisis asistida por microondas. Este hecho deriva en la liberación de mayores concentraciones de monosacáridos en la hidrólisis enzimática con valores comprendidos entre 43.8 y 65.3 g/L. Por lo tanto, se pone de manifiesto la importancia de una correcta deslignificación en el pretratamiento para facilitar la accesibilidad a la celulosa y la hemicelulosa a las enzimas en la hidrólisis.

VIII. La utilización de la relación butanol-biomasa, variable que cuantifica la conversión de paja de arroz en biobutanol, permite evaluar conjuntamente el pretratamiento, la hidrólisis enzimática y la fermentación. Al usar esta variable como respuesta en la optimización del pretratamiento alcalino se ha observado que la configuración que maximiza la concentración de monosacáridos liberados en la etapa de hidrólisis se correlaciona con aquella que maximiza la producción de butanol, pero subestima la conversión de biomasa en butanol en un 13% al no considerar otros factores como la solubilización del material durante el pretratamiento.

IX. Se ha observado que las variables del pretratamiento alcalino que muestran mayor efecto sobre la conversión de paja de arroz en biobutanol son la carga de sólidos y la concentración de hidróxido sódico en comparación con otras variables como la temperatura y el tiempo de reacción. Las condiciones óptimas del pretratamiento (134 °C, 20 minutos, hidróxido sódico al 0.75% p/v y carga de sólidos del 5% p/v) permiten alcanzar en el proceso SHF una conversión de biomasa en butanol de 78 gramos por kilogramo de material original tras 72 horas de fermentación junto con una producción de butanol de 10.1 g/L y una productividad de butanol de 0.140 g/L h.

X. Los resultados muestran que la utilización del tampón citrato en la etapa de hidrólisis enzimática afecta negativamente a la posterior etapa fermentativa, obteniéndose producciones de butanol inferiores a las obtenidas con el tampón acetato.

XI. La microscopía electrónica de barrido ha permitido observar que la cepa bacteriana *C. beijerinckii* DSM 6422 se inmoviliza en la superficie de la paja de arroz pretratada, pero no forma una estructura de biopelícula. A lo largo del proceso SSFR en modo semicontinuo se ha detectado el incremento de la viscosidad del caldo de cultivo y el hinchamiento de las células bacterianas, sugiriendo la aparición de la autolisis promovida por esporulación como estrategia de supervivencia de la bacteria. Este hecho y la incapacidad de incrementar la carga de sólidos ponen de manifiesto la necesidad de aplicar una estrategia de purga para extender el tiempo operacional del proceso.

Los resultados derivados de esta tesis doctoral han demostrado que el proceso SSF es una configuración eficaz en la producción de biobutanol a partir de paja de arroz, un residuo lignocelulósico no utilizado hasta la fecha en dicho proceso. Se ha profundizado en la mejora de las condiciones de operación, de la composición del medio de fermentación y del pretratamiento. La productividad del proceso se incrementó mediante la integración de la recuperación *in situ* de producto y del modo de alimentación semicontinuo, resultando en una configuración novedosa en la producción de biobutanol a partir de residuos lignocelulósicos. En este sentido, a partir de la información obtenida, se proponen las siguientes líneas de trabajo futuro:

I. Optimización del pretratamiento de hidrotermólisis asistida por microondas, considerando la utilización de hidróxido sódico en dicho método.

II. Evaluación del proceso SSFR en modo semicontinuo a mayor escala. Puesta en marcha de una planta piloto como un escenario más próximo a condiciones de operación industrial.

III. Estudio del proceso SHF en modo de alimentación continuo con la finalidad de incrementar la productividad de biobutanol.

10. RESUMEN EXTENDIDO

El conocimiento de los efectos adversos del cambio climático sobre la salud humana y el medioambiente ha propiciado en las últimas décadas una profunda transformación del sistema energético global en la que toma especial relevancia el uso de energías renovables. En ese contexto, el Pacto Verde Europeo pretende acabar con las emisiones netas de gases de efecto invernadero en los Estados Miembro de la Unión Europea en 2050, enmarcándose en una prometedora agenda ambiental que incluye objetivos a corto plazo como la consecución en 2030 de una cuota mínima en el transporte del 3.5% de una serie de biocombustibles entre los que se encuentran los biocarburantes producidos a partir de residuos lignocelulósicos. Uno de los biocarburantes con mayor potencial para sustituir a los combustibles fósiles es el biobutanol, que puede ser obtenido mediante la fermentación aceto-butílica-etílica (ABE) en la que las bacterias del género *Clostridium* producen los disolventes (acetona:butanol:etanol) en una proporción másica típica de 3:6:1. En esta fermentación anaerobia pueden utilizarse como sustrato los residuos lignocelulósicos, pero su implantación a nivel industrial está limitada, entre otros factores, por la necesidad de múltiples etapas conectadas en serie: pretratamiento, hidrólisis enzimática, fermentación y recuperación de producto. La finalidad de las etapas previas a la fermentación (pretratamiento e hidrólisis enzimática) es la liberación de los azúcares que constituyen la celulosa y la hemicelulosa, principales componentes de la biomasa lignocelulósica junto con la lignina, ya que estos polisacáridos no son directamente asimilables por parte de las bacterias del género *Clostridium* solventogénicas. El pretratamiento pretende modificar la biomasa lignocelulósica de tal forma que se favorezca la acción de las enzimas en la posterior hidrólisis enzimática, obstaculizada por la presencia de la lignina, entre otros factores. Con el fin de reducir los costes de este proceso multietapa ha aumentado recientemente el interés por el desarrollo de configuraciones integradas como la estrategia de sacarificación y fermentación simultánea (SSF) que combina las etapas de hidrólisis enzimática y de fermentación en un mismo reactor. Esta estrategia permite aumentar la viabilidad económica y la productividad en la obtención de biobutanol a partir de residuos lignocelulósicos y puede reducir la inhibición de la hidrólisis enzimática y de la fermentación por parte del sustrato.

El presente trabajo de tesis doctoral se centra en la evaluación del proceso SSF como una configuración alternativa al proceso convencional de sacarificación y fermentación separada (SHF) en la producción de biobutanol a partir de paja de arroz, así como en la mejora de dicho proceso en base a la optimización de las condiciones de operación y de los componentes del medio de fermentación. La

selección de la paja de arroz como residuo lignocelulósico a valorizar se debe a que está disponible localmente en el Parque Natural de la Albufera (Valencia, España), uno de los humedales más importantes del mediterráneo occidental donde se generan al año entre 75000 y 90000 toneladas de este material. La paja de arroz se quemaba a campo abierto en el parque como práctica habitual, sin embargo, dicha actividad emite gases de efecto invernadero y otros contaminantes que atentan contra la salud humana y la política actual de la Unión Europea exige nuevos modos de gestión. De esta forma, la valorización de la paja de arroz en biobutanol no solo contribuye a lograr los objetivos marcados por la Unión Europea para descarbonizar el sector del transporte, sino que también supone una alternativa de gestión atractiva que puede reducir el impacto de la producción de arroz sobre el medioambiente. En la actualidad existen numerosos estudios de laboratorio en los que se ha aplicado el proceso SSF en modo discontinuo a residuos lignocelulósicos, sin embargo, dicho proceso no se ha aplicado a la paja de arroz hasta la fecha. Atendiendo a la necesidad de pretratar la paja de arroz como paso previo a su uso en el proceso SSF, en esta tesis doctoral se aborda también el estudio de dos pretratamientos: uno más común como es el pretratamiento alcalino con hidróxido sódico y otro método emergente como es la hidrotermólisis asistida por microondas. Por otra parte, el proceso SSF continúa presentando una serie de desafíos como la inhibición de la fermentación por parte del butanol, cuya hidrofobicidad afecta negativamente a las funciones vitales de las bacterias e impide alcanzar concentraciones finales de este disolvente superiores a valores cercanos al 1% (p/v). En este sentido, con el fin de superar estos desafíos y promover la implantación industrial de este proceso en la gestión de la paja de arroz, en esta tesis doctoral se desarrolla una configuración novedosa que combina el proceso SSF y la recuperación *in situ* de producto y que opera en modo de alimentación semicontinuo. Con esta configuración se pretende incrementar la productividad de biobutanol mediante el uso de mayores cargas de biomasa sin que se produzca inhibición por sustrato ni por producto.

En esta tesis doctoral, la paja de arroz pretratada a partir de los métodos estudiados (pretratamiento alcalino con hidróxido sódico o hidrotermólisis asistida por microondas) se hidrolizó empleando la mezcla de celulasas y hemicelulasas Cellic® CTec2 comercializada por la empresa Novozymes. La cepa bacteriana utilizada en la fermentación fue *Clostridium beijerinckii* DSM 6422. La metodología empleada en la planificación de los ensayos se basó en el diseño de experimentos. Esta técnica estadística se aplicó en este trabajo, generalmente, en dos etapas: la identificación de las variables independientes clave sobre la variable respuesta a partir de un diseño factorial fraccionado (FFD) y la maximización de dicha variable

respuesta a partir del modelo de regresión obtenido en un diseño central compuesto (CCD). Para la creación de estos diseños y el posterior análisis estadístico de los datos se usó el programa MINITAB®.

El primer estudio que se presenta en este trabajo consistió en la evaluación del proceso SSF y su comparación con el proceso SHF en el que, a diferencia de la configuración simultánea, la hidrólisis enzimática y la fermentación se llevan a cabo secuencialmente en dos recipientes distintos. En ambos casos, la fermentación tuvo lugar en botellas de suero de 50 mL. La paja de arroz utilizada se pretrató mediante hidrotermólisis asistida por microondas con una carga de sólidos del 10% (p/v) y a 200 °C de temperatura durante 15 minutos. Se aplicaron dos FFD para analizar los efectos del pH inicial (con valores de 5.2 y 6.2 en SSF y de 6.4 y 7.4 en SHF), de la concentración de extracto de levadura (con valores de 2 y 4 g/L) y de la concentración de hierro (con valores de 0.01 y 0.02 g/L) en el medio de fermentación sobre la concentración de butanol producido a las 48 horas de fermentación. Adicionalmente, en el proceso SSF se analizó también el efecto de la carga de enzimas (con valores de 4.1 y 12.4 unidades de papel de filtro -FPU- por gramo de biomasa pretratada) y en el proceso SHF se analizó el efecto del tampón empleado en la hidrólisis enzimática (acetato y citrato 50 mM). Los resultados obtenidos mostraron que las variables con mayor efecto sobre la producción de butanol en el proceso SSF fueron el pH inicial y la carga enzimática, ambas con un efecto positivo. La única variable con un efecto significativo en el proceso SHF fue el tipo de tampón empleado, inhibiéndose la fermentación en aquellos ensayos en los que se utilizó tampón citrato 50 mM, al contrario que en los que se usó tampón acetato 50 mM. A su vez, se observó que en el proceso SSF tanto la producción de butanol (5.2 g/L) como la conversión de biomasa en butanol (48 g/kg de material original) a las 48 horas aumentaron en un 8% respecto a los valores obtenidos en el proceso SHF al mismo tiempo de fermentación (4.9 g/L y 45 g/kg de material original). La conversión de biomasa en butanol se cuantificó con la relación butanol-biomasa, un parámetro que tiene en cuenta el balance de masas de las tres etapas del proceso (pretratamiento, hidrólisis enzimática y fermentación). La productividad total de butanol se incrementó en un 173% en la configuración simultánea (0.109 g/L h) en comparación con la configuración separada (0.040 g/L h), considerando también en este caso el tiempo requerido para llevar a cabo la hidrólisis enzimática (72 horas). En estos ensayos y en todos los posteriores ensayos de la tesis doctoral se observó que la cepa *C. beijerinckii* DSM 6422 no produce concentraciones de etanol detectables por cromatografía de gases o cromatografía de líquidos de alta resolución, obteniéndose una proporción promedio de los disolventes (acetona:butanol) de 1:1.6. En base a los resultados obtenidos en el estudio

comparativo, se demostró que el proceso SSF era una configuración viable en la producción de biobutanol a partir de paja de arroz y que, además, era más eficiente que el proceso SHF. Por otra parte, considerando el efecto que presentaron las variables estudiadas en los FFD, se estableció para todos los posteriores ensayos de la tesis doctoral una concentración de sulfato de hierro de 0.02 g/L en el medio de fermentación y el uso del tampón acetato 50 mM en la etapa separada de hidrólisis enzimática. A continuación, se llevó a cabo una optimización del proceso SSF mediante un CCD en el que se maximizó la producción de butanol a las 48 horas. Las variables a optimizar fueron la carga enzimática con un intervalo de variación comprendido entre 5.4 y 19.1 FPU/g y el pH inicial con un intervalo de variación comprendido entre 5.6 y 7.2. Adicionalmente, también se incluyó en el CCD la concentración de extracto de levadura en un rango más amplio que el estudiado en el FFD (0.5 – 5.5 g/L). Las dos variables con un efecto significativo sobre la respuesta fueron la carga enzimática y la concentración de extracto de levadura, siendo la carga enzimática la variable que presentó un mayor efecto. El experimento de validación, en el que se utilizó el valor óptimo de la carga enzimática (13.5 FPU/g), el valor óptimo de la concentración de extracto de levadura (4.7 g/L) y un pH inicial de 6.4, demostró la correcta capacidad de predicción del modelo de regresión de segundo orden obtenido y resultó en una producción de butanol (disolventes ABE) a las 48 horas de 5.5 g/L (8.4 g/L) junto con una productividad de butanol (disolventes ABE) de 0.114 g/L h (0.175 g/L h) y una conversión de biomasa en butanol de 51 g/kg de material original. Cabe destacar que el 93% de la concentración final de butanol se alcanzó a las 24 horas, resultando en una productividad de 0.212 g/L h. Los resultados de este primer estudio indicaron que, independientemente del proceso aplicado, la producción de butanol y la conversión de biomasa en butanol estaban limitados por la escasa concentración de monosacáridos liberados en la hidrólisis enzimática de la paja pretratada (valores comprendidos entre 23.0 y 26.5 g/L), lo que se relacionó con la reducida eliminación de lignina (13%) y de cenizas (14%) en el pretratamiento con microondas. La limitación de la valorización de la paja de arroz en biobutanol a causa de la relativa inaccesibilidad de la celulosa y de la hemicelulosa de la biomasa pretratada a las enzimas también fue indicada por los elevados valores del rendimiento de butanol en base a los monosacáridos consumidos (0.22 – 0.31 g/g), lo que demostró que la cepa bacteriana metabolizó correctamente los monosacáridos liberados.

En el siguiente estudio se evaluó el pretratamiento alcalino como método alternativo al pretratamiento por hidrotermólisis asistida por microondas, empleando hidróxido sódico con el fin de mejorar la deslignificación de la paja de arroz y, por ende, la accesibilidad de la celulosa y la hemicelulosa a las enzimas. En

este segundo estudio se empleó la configuración SHF, en la que los hidrolizados se fermentaron en botellas de suero de 50 mL, con la finalidad de poder analizar los efectos de las variables de pretratamiento sobre las etapas posteriores de hidrólisis enzimática y de fermentación de manera individual. En base a los resultados obtenidos previamente en el aprovechamiento de la paja de arroz pretratada con microondas, en estos ensayos y en el resto de los ensayos de esta tesis doctoral se mantuvieron las condiciones óptimas del medio de fermentación establecidas en el primer estudio. El diseño de experimentos se basó en el uso de la relación butanol-biomasa a las 72 horas de fermentación como variable respuesta. Este diseño consistió en un primer plan experimental en el que se analizó el efecto de la temperatura de reacción (con valores de 121 y 134 °C), del tiempo de reacción (con valores de 10 y 40 minutos), de la concentración de hidróxido sódico (con valores de 0.2 y 2.0% p/v) y de la carga de sólidos (con valores de 5 y 10% p/v) sobre la relación butanol-biomasa aplicando un FFD. Los resultados obtenidos mostraron que aquellas muestras pretratadas con una concentración de hidróxido sódico del 2.0% (p/v) condujeron a altos porcentajes de eliminación de lignina ($80-98\%$) y que era posible alcanzar un porcentaje de deslignificación del 57% con el uso de una concentración de hidróxido sódico de tan solo el 0.2% (p/v), favoreciendo así la viabilidad económica del pretratamiento al reducir los costes asociados al consumo de reactivo. La hidrólisis enzimática de las muestras que presentaron porcentajes de eliminación de lignina adecuados (≥ 57%) resultó en concentraciones de monosacáridos comprendidas entre 33.4 y 65.3 g/L, evidenciando la relación positiva entre la deslignificación de la paja de arroz en el pretratamiento y la liberación de azúcares por parte de las enzimas en la hidrólisis. Tras fermentar los hidrolizados, la máxima concentración de butanol (10.6 g/L) se obtuvo a partir del hidrolizado con más azúcares disponibles en un tiempo de reacción de 72 horas. La presencia de ácidos no disociados (ácido acético y ácido butírico) en concentraciones cercanas a 23 mM a las 24 horas de fermentación afectó negativamente a la conversión de azúcares en butanol, por lo que el pH inicial se fijó en 5.8 para posteriores fermentaciones con la finalidad de limitar la concentración de ácidos no disociados. La única variable con un efecto significativo sobre la relación butanol-biomasa a las 72 horas de fermentación fue la carga de sólidos, presentando un efecto negativo, por lo que se fijó en un valor del 5% (p/v) en el pretratamiento de paja de arroz. Por otra parte, se seleccionó la temperatura de 134 °C porque permitió disminuir la concentración de hidróxido sódico sin que se viese afectada la conversión de biomasa en butanol. Como etapa final del diseño de experimentos se realizó una optimización de las variables cuantitativas (concentración de hidróxido sódico y tiempo de reacción) para maximizar la relación butanol-biomasa empleando un CCD, cuyos valores óptimos se validaron en una

etapa posterior. El rango estudiado fue de 0.0 a 1.0 (% p/v) para la concentración de hidróxido sódico y de 20 a 60 minutos para el tiempo de reacción. Tras hidrolizar enzimáticamente las muestras pretratadas con una concentración de hidróxido sódico igual o superior al 0.5% (p/v), se liberaron concentraciones de azúcares superiores a 50 g/L (valor máximo de 64.4 g/L) que, tras su fermentación, resultaron en producciones de butanol a las 72 horas comprendidas entre 8.1 y 10.3 g/L. Aunque se empleó un rango de concentraciones de hidróxido sódico con un valor máximo de 1.0% (p/v), menor al valor máximo empleado en los ensayos del FFD (2.0% p/v), se llegaron a liberar en la hidrólisis enzimática concentraciones de azúcares similares. En referencia a la conversión de biomasa en butanol, la variable que tuvo un efecto significativo sobre la relación butanol-biomasa fue la concentración de hidróxido sódico. Los valores de las variables que maximizaban la conversión de biomasa en butanol (134 °C, 20 minutos, hidróxido sódico al 0.75% p/v y carga de sólidos del 5% p/v) se validaron en un experimento posterior. Los resultados de esta validación fueron una conversión de biomasa en butanol de 78 g/kg de material original tras 72 horas de fermentación junto con una producción de butanol (disolventes ABE) de 10.1 g/L (16.7 g/L) y una productividad de butanol (disolventes ABE) de 0.140 g/L h (0.232 g/L h), resultando en una variación de únicamente el 7% respecto a la respuesta predicha por el modelo. El análisis integrado de las tres etapas del proceso (pretratamiento, hidrólisis enzimática y fermentación) mediante la utilización de la relación butanol-biomasa fue una decisión clave para no subestimar la conversión de paja de arroz en butanol, ya que las condiciones que maximizaban la liberación de monosacáridos y la producción de butanol conllevaban su reducción en un valor del 13% al no considerar la pérdida de biomasa en el pretratamiento.

Tras demostrar que el proceso SSF puede ser una alternativa potencial en la gestión de la paja de arroz mediante su conversión a biobutanol y mejorar el pretratamiento de este residuo lignocelulósico, el último estudio de este trabajo se centró en desarrollar un proceso de sacarificación y fermentación simultánea con recuperación *in situ* de producto (SSFR) en modo de alimentación semicontinuo. En un primer plan experimental se optimizó mediante un CCD el proceso SSF en modo discontinuo para paja de arroz pretratada con hidróxido sódico, utilizando las condiciones óptimas de pretratamiento establecidas en el segundo estudio de la tesis doctoral. Los ensayos se llevaron a cabo en botellas de suero de 50 mL. En este caso las variables independientes fueron la carga de sólidos con un rango de 3.8 a 12.2% (p/v) y la carga enzimática con un rango de 3.7 a 26.3 FPU/g. El objetivo fue maximizar la producción de butanol a las 24 horas para favorecer la velocidad de

eliminación de este disolvente del caldo de fermentación mediante arrastre por gas. Ambas variables tuvieron un efecto significativo sobre la respuesta, siendo mayor el efecto de la carga de sólidos. Los valores óptimos fueron 9.2% (p/v) para la carga de sólidos y 19.9 FPU/g para la carga enzimática. Tras la optimización, se realizó una etapa de validación de las condiciones óptimas, obteniéndose una concentración de butanol (disolventes ABE) de 9.3 g/L (15.5 g/L) a las 24 horas y de 11.9 g/L (19.4 g/L) a las 72 horas, lo que resultó en una productividad de butanol de 0.165 g/L h y de disolventes ABE de 0.270 g/L h junto con una conversión de biomasa en butanol de 63 g/kg de material original. La comparación de los resultados obtenidos en los procesos SHF y SSF llevados a cabo a partir de paja de arroz pretratada con hidróxido sódico mostraron un incremento del 136% en la productividad total de butanol con la configuración simultánea, volviendo a poner de manifiesto la elevada eficiencia del proceso SSF en comparación con el proceso SHF. Así mismo, al comparar estos resultados con los obtenidos en el proceso SSF a partir de paja de arroz pretratada con microondas, se observó un incremento del 117% en la producción de butanol y del 23% en la conversión de biomasa en butanol, lo que demostró la importancia de utilizar un pretratamiento adecuado. Las condiciones establecidas en este primer plan experimental se aplicaron en el cambio de escala del proceso SSF en modo discontinuo a un volumen de trabajo de 500 mL a partir de un reactor de tanque agitado de 2 litros. Se obtuvieron producciones de butanol de 9.1 y 12.2 g/L a las 24 y 72 horas, respectivamente, observándose diferencias menores al 3% en comparación con el experimento llevado a cabo en botellas de suero. En ambos ensayos, el cese en la producción de butanol y el incremento en la concentración de monosacáridos en el medio entre las 48 y 72 horas señalaron la inhibición de la fermentación a concentraciones de butanol cercanas a 12 g/L, lo que puso de manifiesto la importancia de limitar la concentración de butanol en el medio con el fin de incrementar la biomasa procesada como vía para aumentar la productividad. En la última experimentación de esta tesis doctoral se evaluó el proceso SSFR en modo semicontinuo, utilizando el arrastre por gas como operación unitaria en la recuperación *in situ* de producto. Para ello se diseñó y se puso en marcha un dispositivo experimental que contenía un reactor de tanque agitado de 2 litros, un sistema de recirculación para burbujear los gases producidos en la fermentación (hidrógeno y dióxido de carbono) con un caudal volumétrico de 4 L/min y un condensador mantenido a 4 °C para recuperar los disolventes arrastrados a la corriente gaseosa. La alimentación del sustrato a lo largo de la operación se realizó mediante tres adiciones, el 50% de la paja de arroz pretratada se añadió al inicio del experimento (con una carga de sólidos del 9.2% p/v) y el 50% restante se añadió a las 20 y a las 30 horas en dos alimentaciones adicionales de la misma carga, resultando en una carga final de sólidos del 18.4% (p/v). El arrastre por gas comenzó

tras la primera alimentación adicional de biomasa y se operó durante 30 horas para mantener una concentración de butanol en el medio comprendida entre 5 y 12 g/L. El plan de experimentación se diseñó también para analizar el efecto que tiene una reducción de la carga enzimática y de los componentes del medio (compuestos tampón, extracto de levadura y minerales) sobre la producción acumulativa de biobutanol con la finalidad de reducir los costes de operación del proceso. Para ello se llevaron a cabo tres experimentos. En el primero, la carga enzimática se mantuvo en el valor óptimo de 19.9 FPU/g a lo largo de toda la operación mediante la adición de la mezcla enzimática junto a la alimentación de la biomasa. En los otros dos experimentos, únicamente se añadieron las enzimas al inicio de la operación, por lo que la carga enzimática final se redujo a la mitad (10.0 FPU/g). Respecto a la reducción de los componentes del medio, en los dos primeros experimentos el medio se suplementó junto con las alimentaciones adicionales de sustrato, manteniendo de esta forma la misma relación entre la biomasa añadida y la concentración de los componentes del medio que la fijada al inicio del proceso. En el tercer experimento, el medio solo se suplementó al inicio. La combinación del proceso SSF con la recuperación *in situ* de producto y el modo de alimentación semicontinuo permitió duplicar, respecto a los valores obtenidos en el proceso discontinuo, la producción de butanol (disolventes ABE) a un valor de 24.8 g/L (38.5 g/L) y su productividad a 0.344 g/L h (0.534 g/L h) durante las 72 horas en las que se operó el sistema. De esta forma, no solo se demostró que la aplicación de esta configuración avanzada en la producción de biobutanol a partir de residuos lignocelulósicos era técnicamente factible, sino que también se demostró que aumentaba la productividad del proceso. Los resultados de la recuperación de butanol mediante el arrastre por gas mostraron una baja selectividad de este disolvente (aproximadamente 5), lo que sugirió la necesidad de mejorar esta operación unitaria. Por otra parte, la observación de las muestras de biomasa mediante la técnica de microscopía electrónica de barrido mostró que la bacteria *Clostridium beijerinckii* DSM 6422 se inmovilizó en la superficie de la biomasa pretratada tras 20 horas sin formar una estructura de biopelícula. Tras 50 horas de operación, la densidad celular disminuyó y algunas de estas bacterias presentaron una morfología hinchada, lo que sugirió, junto con el hecho de que el medio se volvió más viscoso, la formación de esporas. La esporulación de las bacterias *Clostridium* pudo impedir el progreso de la fermentación tras una tercera alimentación adicional de biomasa a las 72 horas, sugiriendo la necesidad de aplicar una estrategia de purga. Los resultados de los experimentos llevados a cabo para analizar la influencia de la reducción de los reactivos empleados en el proceso mostraron que la reducción a la mitad de la carga enzimática no presentó un efecto negativo sobre la producción y la productividad de butanol. En cambio, la reducción

de compuestos tampón, extracto de levadura y/o minerales sí que afectó negativamente al proceso con una reducción del 20% en la producción acumulativa de butanol, observándose para este caso una disminución del pH a las 30 horas.

11. ACRÓNIMOS

ABC: *ATP-Binding Cassette*

ABE: *Acetone-Butanol-Ethanol*

ANOVA: *Analysis of Variance*

CBP: *Consolidated Bioprocessing*

CCD: *Central Composite Design*

CCR: *Carbon Catabolite Repression*

CRISPR: *Clustered Regularly Interspaced Short Palindromic Repeats*

CSTR: *Continuous Stirred Tank Reactor*

DAD: *Diode Array Detector*

DOE: *Design of Experiments*

DSMZ: *Deutsche Sammelstelle für Mikroorganismen und Zellkulturen*

EMP: *Embden-Meyerhoff-Parnass*

ETBE: *Ethyl-Tert-Butyl-Ether*

FAME: *Fatty Acid Methyl Ester*

FFD: *Fractional Factorial Design*

FPU: *Filter Paper Unit*

GAE: *Gallic Acid Equivalents*

GC: *Gas Chromatography*

GHG: *Greenhouse Gases*

HMF: *Hydroxymethylfurfural*

HPLC: *High Performance Liquid Chromatography*

IC: *Ion Chromatography*

IEA: *International Energy Agency*

IPCC: *Intergovernmental Panel on Climate Change*

ISPR: *In Situ Product Recovery*

MTBE: *Methyl-Tert-Butyl-Ether*

NREL: *National Renewable Energy Laboratory*

OD_{600}: *Optical Density at 600 nm*

PBR: *Packed Bed Reactor*

PDMS: *Polydimethylsiloxane*

PKP: *Phosphoketolase Pathway*

POMS: *Polyoctylmethylsiloxane*

PPP: *Pentose Phosphate Pathway*

PTS: *Phosphotransferase System*

RCM: *Reinforced Clostridial Medium*

RID: *Refractive Index Detector*

RNA: *Ribonucleic Acid*

RS: *Rice Straw*

RSM: *Response Surface Methodology*

SD: *Standard Deviation*

SEM: *Scanning Electron Microscope*

SF: *Severity Factor*

SHF: *Separate Hydrolysis and Fermentation*

SHFR: *Separate Hydrolysis, Fermentation and Recovery*

SRHF: *Simultaneously Repeated Hydrolysis and Fermentation*

SSF: *Simultaneous Saccharification and Fermentation*

SSFR: *Simultaneous Saccharification, Fermentation and Recovery*

STR: *Stirred Tank Reactor*

TFM: *Modified Polytetrafluoroethylene*

UNFCCC: *United Nations Framework Convention on Climate Change*

WHO: *World Health Organization*

WRI: *World Resources Institute*

12. ANEXO

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Full Length Article

Comparison of simultaneous saccharification and fermentation and separate hydrolysis and fermentation processes for butanol production from rice straw

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ABSTRACT

Rice straw (RS) is one of the lignocellulosic wastes with the highest global production. The main objective of this study was to maximise the butanol production by *Clostridium beijerinckii* DSM 6422 from RS pretreated by microwave-assisted hydrothermolysis. Two different fermentation strategies were compared: separate hydrolysis and fermentation (SHF, two-step process) and simultaneous saccharification and fermentation (SSF, one-step process). In parallel, the variables that significantly affected the butanol production were screened by using fractional factorial designs. Butanol concentration and productivity at 48 h were, respectively, 8% and 173% higher in SSF than in SHF. A one-step process was more efficient than a two-step process, especially considering the time savings derived from much higher productivity. From these results, SSF was further optimised by response surface methodology with central composite design over the key factors on the butanol production at 48 h: initial pH, enzyme loading and yeast extract concentration. The optimum point yielded a butanol productivity of 0.114 g $L^{-1}h^{-1}$, with a butanol-biomass ratio of 51 g kg^{-1} of raw RS (ABE-biomass ratio of 77.0 g kg^{-1} of raw RS). The parameter with the greatest effect was enzyme loading, with an optimal value of 13.5 FPU g-dw^{-1}. This study showed that microwave-processed RS has great potential as a substrate for the butanol production from ABE fermentation when combining process stages by SSF.

1. Introduction

The expected increase of the world population by more than 30% in the next 40 years, the depletion of resources, external energy dependence and climate change are altering the way in which biological resources in Europe are managed. In this context, interest in biomass as a source of carbon and energy has increased [1]. Lignocellulosic material is the most abundant and economical biomass on the planet [2]. Numerous raw materials, such as agricultural residues, forestry wastes, industrial and municipal wastes, and bioenergy crops, are available for the production of biofuels, including biobutanol [3]. However, a pretreatment is necessary to alter the lignocellulosic structure and to remove and/or alter lignin, generally followed by an enzymatic or acid hydrolysis stage to obtain sugar monomers [4].

Biobutanol is mainly produced by *Clostridium acetobutylicum* or *C. beijerinckii* in acetone-butanol-ethanol (ABE) fermentation by a pathway consisting of two metabolic phases: acidogenesis, followed by solventogenesis [5]. During acidogenesis bacterial growth occurs with the production of acids, hydrogen and carbon dioxide; whereas in the solventogenesis stage the production of solvents and endospore formation occurs [6]. These gram-positive and anaerobic bacteria belong to the only genus capable of producing this solvent as a major metabolite [7]. Butanol has some benefits as a fuel in terms of energy density, handling, transport and storage [8]. Despite these advantages, its production by fermentation cannot compete economically with the butanol obtained in the petrochemical industry due to, among other causes, lower development of bioprocesses, long fermentation times, high cost of the substrate, low yields and high cost of product recovery [9]. Strategies developed to enhance cellulosic biobutanol production include strain improvement by genetic engineering, optimisation of the medium formulation and combination of ABE fermentation stages [4]. To screen and optimise the effect of medium conditions and process parameters on ABE productivity, statistical techniques such as fractional factorial design and response surface methodology (RSM) are often used [10].

The processes derived from the combination of ABE fermentation stages are simultaneous saccharification and fermentation (SSF), consolidated bioprocessing (CBP), separate hydrolysis and fermentation

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with *in-situ* recovery (SHFR) and simultaneous saccharification and fermentation with *in-situ* recovery (SSFR) [4]. SSF was developed by Gauss et al. [11] and combines enzymatic hydrolysis and ABE fermentation in one step, increasing the butanol yield and productivity compared to separate hydrolysis and fermentation (SHF). SSF could potentially reduce operational costs and the risk of contamination. In addition, the SSF process minimises glucose inhibition on cellulases and β-glucosidase because bacteria consume sugars as soon as they are released [4]. For example, Qi et al. [12] observed that butanol production was higher in SSF (12.64 g L^{-1}) than in SHF (11.25 g L^{-1}) by fermenting ammonium sulfite-pretreated wheat straw with *C. acetobutylicum* ATCC 824, despite decreasing the biomass loading from 10.5 to 9% (w/v). Not only was SSF more efficient in terms of butanol production and time, but enzyme loading was reduced by one-half, thereby conferring an economic advantage. However, Shao and Chen [13] obtained a shorter fermentation time and a higher butanol concentration by the same bacterial strain from *Amorphophallus* konjac waste in SHF, suggesting that the most appropriate process depends on factors such as the feedstock type and the strain of bacteria used.

One of the most abundant lignocellulosic wastes in the world is rice straw (RS), with an estimated annual production of 731 million tons [14]. Unlike other straws, RS is not generally used as animal feed due to its low digestibility and, apparently, it has a low value for social benefit, so it is burnt openly in the field, causing air pollution [15]. There are numerous reported pretreatments (physical, chemical, physicochemical and biological) to enhance ABE fermentation of RS [16]. Despite the low lignin content in RS [17], these methods must face other limiting factors, such as the presence of accumulated silica [18] and high cellulose crystallinity [19]. Among these pretreatment options, dielectric heating by microwave irradiation is used on lignocellulose as an alternative to convection heating [20]. Indeed, Ma et al. [18] noticed that microwave pretreatment could improve the enzymatic accessibility of cellulose by partially breaking the lignin-hemicellulose structure and the waxy structure of silicon, increasing solubility. Furthermore, Zhu et al. [19] determined that, compared to the alkali-alone process, microwave-assisted alkali pretreatment eliminates more hemicellulose and lignin from RS, consequently obtaining a hydrolysate with more glucose and less xylose after enzymatic hydrolysis. One of the limitations for the production of biobutanol is the generation during pretreatment of compounds that inhibit microbial growth, such as acetic acid, 5-hydroximethylfurfural (HMF) and furfural [16]. After pretreating RS with dilute acid, Hsu et al. [17] observed a correlation between the generation of these compounds and pretreatment severity. Indeed, Fonseca et al. [21] demonstrated that detoxification of rice improved the ethanol productivity from RS hydrolysate with dilute acid. Another alternative to overcome the toxicity derived from chemical pretreatment is the use of non-catalysed methods such as microwave irradiation. This strategy can avoid problems of inhibition by these compounds, saving at the same time the cost derived from chemicals. Although SSF processes have been reported for butanol production by ABE fermentation using other agricultural waste such as wheat straw [8,12,22], corn stover [23] or corncob [24], among others, there is no literature data on the effect of using SSF to produce butanol from RS.

The scope of this work is to evaluate the SSF process for butanol production by *C. beijerinckii* DSM 6422 from RS previously treated by microwave-assisted hydrothermolysis. SSF configuration was compared with SHF in terms of butanol productivity by evaluating the effect of the following parameters: type of buffer (citrate or acetate) and enzyme loading for enzymatic hydrolysis; and initial pH, yeast extract concentration and iron concentration in the fermentation broth in two sets of fractional factorial design experiments. In a later stage, SSF was further optimised using RSM with central composite design (CCD) over variables with statistically significant effects.

2. Materials and methods

2.1. Materials

RS was obtained from local farmers of L'Albufera located near Valencia (Spain). The biomass was dried for 24 h at room temperature, cut into fragments of ~ 2 cm and milled. Particle size between 100 and 500 μm was selected by ISO-3310.1 sieve (CISA, Spain), afterwards it was dried in an oven at 45 °C until the residual moisture content was less than 5% (w/w), and it was then stored for further use. The commercial enzyme blend Cellic® CTec2 (Novozyme, Denmark) was employed for hydrolysis of the pretreated RS. The cellulase activity of the enzyme was measured according to the method of the National Renewable Energy Laboratory (NREL) [25], resulting in a value of 119 filter paper units (FPU) mL^{-1}.

2.2. RS pretreatment

Microwave-assisted hydrothermal hydrolysis was performed in an ETHOS One microwave digestion system (Milestone, Italy). The microwave had a maximum power of 1500 W and was controlled via a microprocessor with a capacity of 10 TFM vessels (an internal temperature sensor was installed in a reference vessel). The RS was pretreated at 10% (w/v) using 3 g of dry biomass in 30 mL of deionized water. The microwave was heated using the following ramp of temperature: an initial increase to 100 °C at a rate of 15 °C min^{-1}, which was then increased at 6 °C min^{-1} until 160 °C and then to 4 °C min^{-1} until 200 °C, holding at 15 min [26]. Once the heating was finished, the vessels were cooled at room temperature. The slurry was centrifuged at 10000 rpm for 5 min (centrifuge 5804, Eppendorf, Germany), and the solid phase was washed with deionized water and pH was adjusted to 6.5. Finally, the pretreated RS was dried at 45 °C.

2.3. Microorganism and inoculum preparation

The bacterial strain *Clostridium beijerinckii* DSM 6422 (NRRL B-592) was supplied by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The strain was stored at −80 °C in a Reinforced Clostridial Medium (RCM) with 20% (v/v) glycerol. Before fermentation, the cells were grown in 50-mL serum bottles containing 40 mL of modified RCM (19 g L^{-1} RCM supplemented with 10 g L^{-1} glucose) under anaerobic conditions by sparging pure nitrogen in the medium. The inoculum was statically incubated at 37 °C for 24 h. The media used in the cryopreservation and the inoculum preparation were sterilized in an autoclave for 21 min at 121 °C.

2.4. ABE fermentation

2.4.1. ABE fermentation by SHF

Pretreated RS was hydrolysed prior to fermentation in a separate vessel using the commercial enzyme blend Cellic® CTec2. Enzymatic hydrolysis was carried out in a 100-mL conical flask (with 50 mL of working volume) in a SI500 orbital shaker (Stuart, UK). The hydrolysis process was performed at 50 °C and 150 rpm for 72 h with a biomass loading of 10% (w/v) and an enzyme dosing of 4.1 FPU g-dw^{-1}. The buffer employed was citrate (50 mM) or acetate (50 mM), whose effects on ABE fermentation were assessed using the fractional factorial design of the experiment described in section 2.6. The initial pH was adjusted to 5.0 by NaOH and HCl. After enzymatic hydrolysis, the samples were centrifuged (6 min, 4000 rpm), filtered by 1.2 μm and stored at 4 °C for a maximum of 12 h before fermentation. A volume of 34.6 mL of the enzymatic hydrolysate was fermented in 50-mL serum bottles with a working volume of 40 mL. The concentration of the buffer and the minerals was based on a modified P2 medium introduced by Monot et al. [27]: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} NH_4OAc,

0.09 g L^{-1} $MgSO_4{\cdot}7H_2O$ and 0.001 g L^{-1} $MnSO_4{\cdot}H_2O$. The resazurin concentration was set to 1 mg L^{-1}. $FeSO_4{\cdot}7H_2O$ and yeast extract were added in concentrations of 0.01 or 0.02 g L^{-1} and 2 or 4 g L^{-1} respectively, and the initial fermentation pH was adjusted to 6.4 or 7.4, according to the fractional factorial design of the experiment. Beforehand, the sealed bottles were autoclaved for 10 min at 121 °C; the oxygen was displaced by sparging pure nitrogen. The inoculation was carried out with 2 mL (5% v/v) of actively growing cells, and the serum bottles were incubated at 37 °C and 150 rpm for 72 h.

2.4.2. ABE fermentation by SSF

In this configuration, the pretreated RS was simultaneously hydrolysed and fermented in a 50-mL serum bottle (working volume of 40 mL) with a biomass loading of 9% (w/v). The medium for conducting the SSF experiments was the same as that for the SHF experiments, except that no hydrolysis buffer (50 mM citrate or acetate) was added as the fermentation media contained 28.5 mM of acetate. The effect of the same media parameters (iron and yeast extract concentrations) as in SHF was assessed by the fractional factorial design of the experiment. In this case, the initial reaction pH was set to 5.2 or 6.2 as representatives of optimum values for saccharification or fermentation respectively. The oxygen was displaced by sparging pure nitrogen before autoclaving for 10 min at 121 °C. Afterwards, the enzyme was added along with the inoculum. A loading of 4.1 or 12.4 FPU g-dw^{-1} of Cellic® CTec2 was used in the fractional factorial design experiments in order to assess its influence in SSF. The inoculation was carried out with 2 mL (5% v/v) of actively growing cells. The SSF bottles were incubated at 37 °C and 150 rpm for 120 h. Additionally, two independent replicates of a control experiment (without inoculation) were carried out with the maximum enzyme loading (12.4 FPU g-dw^{-1}) at the minimum pH (5.2) in order to evaluate the maximum release of monosaccharides from the pretreated RS. From results obtained as described herein, CCD was used for further optimisation of the SSF results.

2.5. Analytical methods

The structural carbohydrates, lignin and the moisture content of the RS were determined according to the National Renewable Energy Laboratory (NREL) procedures [28]. The characterisation of the fermentation was carried out by the analysis of pH, cell growth, production of acids and solvents, and sugar uptake from 1-mL samples collected at appropriate times. The pH was measured by a Minitrode electrode (Hamilton, USA). Cell density (g-dw L^{-1}) was calculated from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany). The correlation between OD_{600} and cell density was determined as follows: g-dw L^{-1} = 0.2153·OD_{600} + 0.0689 (n = 10, R^2 = 0.9907). Samples were centrifuged at 10000 rpm for 5 min, and the supernatant was filtered by 0.22 μm before chromatographic analysis. Acids (acetic acid and butyric acid) and solvents (butanol, acetone and ethanol) were analysed in a gas chromatograph (TRACE GC Ultra, Thermo Scientific, USA) equipped with a Teknokroma TRB-FFAP capillary column (30 m × 0.25 mm × 0.25 μm), with helium as carrier gas at a flow rate of 1 mL min^{-1}. One microliter of acidified samples was injected at 250 °C (10:1 split ratio), and the compounds were detected in a flame ionization detector at 250 °C. The oven temperature was held at 50 °C for 4 min, increased at 30 °C min^{-1} until 80 °C (hold time 3 min), and increased at 20 °C min^{-1} until 210 °C (hold time 5 min). Sugars (glucose, xylose and arabinose) were analysed by an ion chromatograph (883 Basic IC plus, Metrohm, Switzerland) equipped with an amperometric detector and a Metrosep Carb 2 anion exchanger column (150 mm × 4 mm × 5 μm). The mobile phase (20 mM NaOH) was set at a flow rate of 0.5 mL min^{-1}. Data are the mean of, at least, two technical replicates.

For the evaluation of the process performance, the following parameters were used:

$$\text{Butanol (or ABE)} - \text{biomass ratio(g kg}^{-1}) = \frac{\text{Butanol (or ABE) produced (g)/V}_{\text{hydrolisate fermented}}\text{ (L)}}{[\text{Biomass loading (kg L}^{-1})/\text{Solid recovery (\%)}] \times 100} \quad (1)$$

$$\text{Butanol (or ABE) productivity (g L}^{-1}\text{h}^{-1}) = \frac{\text{Butanol (or ABE) concentration (g L}^{-1})}{\text{Total reaction time (h)}} \quad (2)$$

$$\text{Butanol (or ABE) yield (g g}^{-1}) = \frac{\text{Butanol (or ABE) concentration (g L}^{-1})}{\text{Sugar consumed (g L}^{-1})} \quad (3)$$

To compare the reaction time of the SHF process with that of SSF, the total reaction time of hydrolysis plus fermentation was assessed. Solid recovery refers to biomass recovered after pretreatment expressed as a percentage.

2.6. Design of experiments and statistical analysis

In this work, SSF was first assessed and compared with SHF, by fractional factorial designs. As the SSF process performed better than the SHF process, the significant variables for SSF were further optimised by RSM using CCD. The response variable in all cases was the concentration of butanol produced at 48 h. The commercial software MINITAB® v.18.1 (LEAD Technologies, Inc.) was used for the design of experiments, regression analysis and analysis of variance (ANOVA) at a confidence level of 95% (p-value less than 0.05).

2.6.1. Fractional factorial design and data analysis

A 2^{4-1} fractional factorial design (resolution IV, 8 experiment runs) was used to identify the significant factors affecting butanol production at 48 h both in SHF and SSF processes. The effects of three variables (low level and high level) – yeast extract concentration (2 and 4 g L^{-1}), $FeSO_4{\cdot}7H_2O$ concentration (0.01 and 0.02 g L^{-1}) and initial fermentation pH (6.4 and 7.4 for SHF, and 5.2 and 6.2 for SSF, respectively) – were evaluated in both processes. For SHF, the fourth variable was the buffer employed for enzymatic hydrolysis (50 mM acetate and 50 mM citrate), whereas for SSF it was the enzyme loading (4.1 and 12.4 FPU g-dw^{-1}).

2.6.2. Central composite design and data analysis

After identification of significant factors, an RSM with CCD was used in the SSF process to determine the optimal combination of enzyme loading, initial pH and yeast extract concentration for maximising butanol production. The established range for each factor was as

Table 1
5-Level CCD of 3 variables for the SSF process. α = 1.68.

Independent variables		Coded and real values				
		Level -α	Level −1	Central point (0)	Level + 1	Level + α
Z_1	Enzyme loading (FPU g-dw^{-1})	5.4	8.2	12.2	16.3	19.1
Z_2	Initial pH	5.6	5.9	6.4	6.9	7.2
Z_3	Yeast extract (g L^{-1})	0.5	1.5	3.0	4.5	5.5

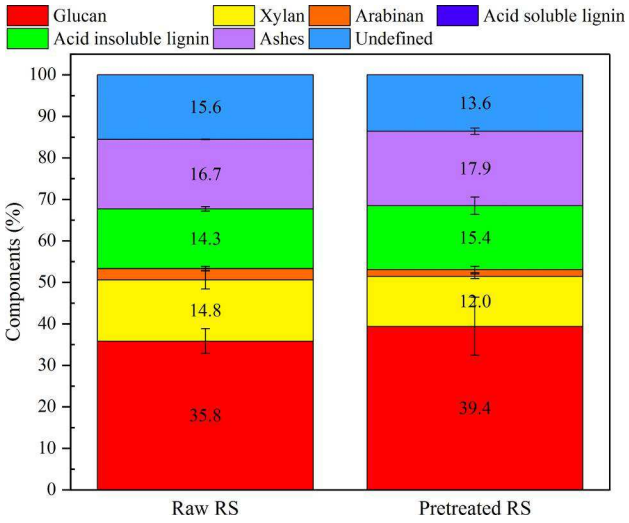

Fig. 1. Chemical composition of raw and pretreated rice straw.

follows: enzyme loading (from 5.4 to 19.1 FPU g-dw^{-1}), initial pH (from 5.6 to 7.2) and yeast extract concentration (from 0.5 to 5.5 g L^{-1}). Table 1 summarises the coded and real values of the three variables used in CCD, which comprised a total of 20 experimental runs with 6 central point replications. Finally, a validation step was carried out by three replicates using the optimised conditions for butanol production.

3. Results and discussion

3.1. Pretreatment of RS

Microwave-assisted hydrothermolysis was selected as RS pretreatment since it presents short reaction times, uniform and rapid heating of biomass, lower generation of inhibitory compounds, higher removal of acetyl groups in hemicellulose, and lower costs in comparison with acid or alkaline pretreatments [29]. The chemical compositions of the raw and pretreated RS are presented in Fig. 1. The untreated dried material consisted of 35.8 ± 2.1% glucan, 14.8 ± 1.6% xylan, 2.7 ± 0.4% arabinan, 0.1 ± 0.0% acid soluble lignin, 14.3 ± 0.4% acid insoluble lignin and 16.7 ± 0.1% ash. This composition is in the typical value range found for RS of different sources [30,31]. Recently, Passoth and Sandgren [32] reported that the typical values for the three major polymers ranged from 29.2 to 34.7% for cellulose, 12.0 to 29.3% for hemicellulose, and 17.0 to 19.0% for lignin, being silica the major ash component.

The pretreatment resulted in a solid recovery of 80.5% of the raw RS with different degrees of degradation among carbohydrate fractions. For example, the glucan percentage increased from 35.8 to 39.4%, although the total percentage of carbohydrates remained almost stable at ~ 53% due to the loss of hemicellulose. During pretreatment only 11.5% of glucan was lost, but greater degradation of arabinan and xylan was observed, with losses of 50.6% and 34.5% respectively. These results indicated that some hemicellulose was removed in the microwave pretreatment of RS; phenomena also observed by Zhu et al. [19] at lower irradiation powers. Higher values of polysaccharides were recovered from raw RS (80.1%) when compared to previous studies on this type of biomass. For example, Amiri et al. [33] obtained a recovery of 76.2% after organosolv pretreatment with a 75% (v/v) ethanol and 1% (w/w) sulfuric acid solution at 150 °C during 30 min, and Moradi et al. [34] found a value of 51.9% after 3 h of alkaline pretreatment at 0 °C with a 12% (w/v) NaOH solution. The microwave irradiation at the tested conditions resulted in a 13.3% delignification and removed 13.7% of ashes (silica content), thus improving the RS digestibility. However, the delignification degree obtained in this study could impact the saccharification of the waste, since lignin binds non-productively to cellulase due to its hydrophobic nature [35]. On the other hand, the remaining silica in the pretreated RS could also act as a physical barrier, protecting for enzymatic hydrolysis [36].

Table 2
2^{4-1} fractional factorial design and experimental results for the SHF and for the SSF processes.

SHF Process

Run	Real values				Response
	Yeast extract (g L^{-1})	$FeSO_4{\cdot}7H_2O$ (g L^{-1})	Initial pH	Saccharification buffer	Butanol 48 h (g L^{-1})
1	2	0.01	6.4	50 mM acetate	4.68
2	2	0.02	6.4	50 mM citrate	3.13
3	2	0.02	7.4	50 mM acetate	4.78
4	2	0.01	7.4	50 mM citrate	2.72
5	4	0.02	6.4	50 mM acetate	4.84
6	4	0.01	6.4	50 mM citrate	3.16
7	4	0.01	7.4	50 mM acetate	4.85
8	4	0.02	7.4	50 mM citrate	3.05

SSF Process

Run	Real values				Response
	Yeast extract (g L^{-1})	$FeSO_4{\cdot}7H_2O$ (g L^{-1})	Initial pH	Enzyme loading (FPU g-dw^{-1})	Butanol 48 h (g L^{-1})
1	2	0.01	5.2	4.1	1.55
2	2	0.02	5.2	12.4	1.53
3	2	0.02	6.2	4.1	2.80
4	2	0.01	6.2	12.4	4.98
5	4	0.02	5.2	4.1	1.00
6	4	0.01	5.2	12.4	0.58
7	4	0.01	6.2	4.1	1.28
8	4	0.02	6.2	12.4	5.24

3.2. Comparison of ABE fermentation by SHF and SSF: Screening of key factors

The 2^{4-1} fractional factorial design was conducted for the SHF and SSF processes to evaluate the influence of the selected parameters on butanol production. The experimental design and the response results for both processes are shown in Table 2. The analysis of variance (ANOVA) of the outcomes, with the estimated coefficients and significant levels for the regression model and the evaluated variables for the SHF and SSF processes are shown in supplementary material. In both cases, after 72 h of fermentation the concentrations of butanol and ABE increased less than 1% from 48 h. Therefore, butanol production at 48 h was considered as the response variable. The fast rate of solvent production demonstrated the successful balance between the acidogenic and solventogenic metabolic phases of *Clostridium beijerinckii* DSM 6422 using both the appropriate operational conditions and the adequate biomass pretreatment. Models for SHF and SSF were statistically significant, with p-values lower than 0.05. In addition, the values of the coefficient of determination (R^2: 0.9996 and 0.9997 for SHF and SSF, respectively) and the adjusted coefficient of determination (Adj. R^2: 0.9975 and 0.9976 for SHF and SSF, respectively) were close to 1.0, indicating the goodness of fit of the models.

From the two-step process (SHF) results, among the four variables screened, only the type of buffer used during enzymatic hydrolysis was found to be significant (p-value of 0.0120). The linear coefficient of the buffer factor (low–high level: acetate-citrate) was lower than zero, indicating that the use of citrate buffer during the saccharification step negatively affected butanol production. Citrate buffer at 50 mM is widely used to maintain a pH around 5.0 during enzymatic hydrolysis [33,34,37]. Furthermore, Xue et al. [38] showed that 60 mM citrate

buffer was optimum for ABE fermentation of Jerusalem artichoke stalk with *C. beijerinckii* CC101, lower and higher values decreased solvent production. Contrarily, Liu et al. [39] observed that *C. beijerinckii* NCIMB 8052 did not grow with 50 mM citrate; whereas when acetate was used as a buffer ABE fermentation was not inhibited. In our study, butanol concentrations ranged from 2.72 to 3.16 g L^{-1} by using citrate buffer, while a minimum of 4.68 g L^{-1} was obtained in the experiments with acetate as hydrolysis buffer. Our results corroborated that the use of citrate buffer provokes a negative effect on ABE fermentation of pretreated RS by *C. beijerinckii* DSM 6422. The yeast extract concentration did not show a significant effect in the tested range of 2 to 4 g L^{-1}. Contrarily, a significant impact on the production of butanol by *C. acetobutylicum* MTCC 481 from RS hydrolysate was previously observed, with an optimal concentration of 3 g L^{-1} [40]. Thus, this demonstrates the importance of the preliminary screening of the media composition for each specific lignocellulosic waste and bacterial strain. The non-significant effect of iron on butanol production from RS hydrolysate indicates that the quantity containing the raw material along with the amount from the minimum yeast extract concentration supplied to the fermentation broth is sufficient. Gottumukkala et al. [37] determined that the improvement in solvent production by *C. sporogenes* BE01 after removing mineral supplementation from RS hydrolysate could be due to the presence of these minerals in the raw material. Ranjan et al. [40] also found that iron concentration had no impact on ABE fermentation of RS with *C. acetobutylicum* MTCC 481 supplemented with 3 g L^{-1} of yeast extract. Furthermore, the initial fermentation pH was incorporated into the experimental design since it would affect the biochemical and biophysical characteristics of the solventogenic *Clostridium* spp. [41]. Fermentation pH, together with the rate of acid production, is one potential key factor in the concentration of undissociated acids that can inhibit a correct shift towards solventogenesis [42]. In contrast, fermentation pH was found to be non-significant, likely because the sugar concentration released from enzymatic hydrolysis was not sufficiently high enough to unbalance the rate of acid production.

In the case of the one-step process (SSF), two variables were found to be statistically significant. The initial pH had a great effect on butanol production (p-value of 0.0164). Furthermore, the enzyme loading was also significant (p-value of 0.0277). Based on the coded coefficients of the linear effects, the order of importance was as follows: initial pH (1.2041) > enzyme loading (0.7130). These results show that, for SSF, it is better not to use a value near to the optimum for the saccharification of cellulosic materials as the initial pH. Although enzymatic hydrolysis will proceed slowly, solventogenic shift would be favoured. Even though no interaction between initial fermentation pH and enzyme loading had a significant effect, the need to use a pH above the optimum for enzymatic hydrolysis could explain the higher enzyme loading required in SSF (12.4 FPU g-dw^{-1}) to achieve butanol concentrations above 4 g L^{-1} compared with the SHF process (4.1 FPU g-dw^{-1}). Furthermore, the *in-situ* ABE products in SSF can be linked to the higher enzyme requirements, as they have been shown as inhibitors of the cellulolytic and hemicellulolytic enzyme activity [43]. Contrarily, the fermentation temperature selected (37 °C) has been reported as a more suitable temperature in comparison to 50 °C for better cellulase and xylanase activities in the presence of ABE products [43].

Both configurations were compared in terms of process efficiency.

Table 4
CCD experimental matrix along with the observed and predicted values of the response for the SSF process.

Run	Real values			Butanol (g L^{-1})	
	Z_1	Z_2	Z_3	Observed	Predicted
1	8.2	5.9	1.5	2.62	2.22
2	16.3	5.9	1.5	2.52	2.96
3	8.2	6.9	1.5	2.79	2.70
4	16.3	6.9	1.5	3.68	3.99
5	8.2	5.9	4.5	4.21	4.00
6	16.3	5.9	4.5	4.62	4.82
7	8.2	6.9	4.5	3.83	3.50
8	16.3	6.9	4.5	4.38	4.88
9	5.4	6.4	3.0	1.06	1.72
10	19.1	6.4	3.0	4.31	3.50
11	12.2	5.6	3.0	4.14	4.17
12	12.2	7.2	3.0	4.80	4.62
13	12.2	6.4	0.5	3.11	3.01
14	12.2	6.4	5.5	5.31	5.25
15	12.2	6.4	3.0	n.a.[a]	4.96
16	12.2	6.4	3.0	4.58	4.96
17	12.2	6.4	3.0	5.19	4.96
18	12.2	6.4	3.0	5.10	4.96
19	12.2	6.4	3.0	4.81	4.96
20	12.2	6.4	3.0	5.10	4.96

[a] n.a.: non available

Table 3 summarises the experimental data obtained in the runs with the highest butanol production at 48 h for each configuration (run 7-SHF, run 8-SSF). The values of released sugars (g L^{-1}), butanol and ABE production (g L^{-1}), butanol and ABE yield (g g^{-1}), butanol and ABE-biomass ratio (g kg RS^{-1}), and butanol and ABE productivity (g $L^{-1}h^{-1}$) are included. After 72 h of enzymatic hydrolysis (SHF), the concentrations obtained of glucose, xylose and arabinose were 17.68, 6.10 and 0.39 g L^{-1} respectively. In order to evaluate the maximum sugars released in the SSF processes, two control saccharification assays without inoculum (initial pH = 5.2, enzyme loading = 12.4 FPU g-dw^{-1}) were carried out, with average glucose, xylose and arabinose concentrations of 18.92, 6.91 and 0.64 g L^{-1} respectively. Regardless of the process used, the concentrations of sugars released by the enzyme blend Cellic® CTec2 from the pretreated RS were very similar (~50% sugar recovery). Thus, corroborating the idea that the need for higher enzyme dosing in SSF than in SHF relies on the enzymatic inhibition by ABE products and/or on the impossibility of performing the saccharification at the optimum pH. The delignification (13.3%) combined with the ash removal (13.7%) achieved after microwave pretreatment limited, to some extent, the sugar recovery from enzymatic hydrolysis. Concerning the SHF configuration, the butanol concentration at 48 h was 4.85 g L^{-1} with an ABE concentration of 7.95 g L^{-1} (butanol:acetone mass ratio of 1.6:1, ethanol was not detected). All the released sugars were consumed at the end of the fermentation, resulting in a butanol (ABE) yield of 0.245 g g^{-1} (0.402 g g^{-1}). The RS exploitation was evaluated with the butanol or ABE-biomass ratio, with observed values of 44.6 g of butanol and 73.1 g of ABE per kg of raw RS. In the SSF process, the butanol concentration at 48 h of reaction time (5.24 g L^{-1}) increased by 8% of that observed in SHF and, in turn, the butanol-biomass ratio rose to 48.2 g kg RS^{-1}; whereas the ABE concentration (butanol:acetone mass ratio of 1.8:1, ethanol was not detected) increased only by 3.7%. The

Table 3
Comparison of SHF and SSF processes after 48 h of fermentation.

Method	Released Sugars (g L^{-1})[a]		Butanol (ABE) Production (g L^{-1})	Butanol (ABE) Yield (g g^{-1})	Butanol (ABE)-biomass Ratio (g kg RS^{-1})	Butanol (ABE) Productivity (g $L^{-1}h^{-1}$)
	Glucose	Xylose				
SHF	17.68	6.10	4.85 (7.95)	0.245 (0.402)	44.6 (73.1)	0.040 (0.066)
SSF	18.92	6.91	5.24 (8.24)	0.217 (0.341)	48.2 (75.8)	0.109 (0.172)

[a] Sugars obtained after 72 h of hydrolysis time. In the case of SSF, sugars released from two abiotic controls.

Table 5
ANOVA of the second-order model for butanol production by SSF process.

Source	Degrees of freedom	Sum of squares	Mean square	*F* value	*p*-value Prob > F	Coefficient[a]
Model	9	20.6456	2.2940	9.04	0.0015	
Linear	3	10.1421	3.3807	13.33	0.0012	
Z_1: Enzyme loading	1	3.8221	3.8221	15.07	0.0037	0.5290
Z_2: Initial pH	1	0.2432	0.2432	0.96	0.3531	0.1334
Z_3: Yeast extract	1	6.0768	6.0768	23.95	0.0009	0.6671
Square	3	9.8683	3.2894	12.97	0.0013	
Z_1Z_1	1	9.4120	9.4120	37.10	0.0002	−0.8304
Z_2Z_2	1	0.5516	0.5516	2.17	0.1744	−0.2010
Z_3Z_3	1	1.1750	1.1750	4.63	0.0598	−0.2934
2-way interactions	3	0.6353	0.2118	0.83	0.5079	
Z_1Z_2	1	0.1591	0.1591	0.63	0.4488	0.1410
Z_1Z_3	1	0.0035	0.0035	0.01	0.9086	0.0210
Z_2Z_3	1	0.4726	0.4726	1.86	0.2054	−0.2431
Error	9	2.2832	0.2537			
Lack-of-fit	5	2.0244	0.4049	6.26	0.0500	
Pure error	4	0.2588	0.0647			
Total	18	22.9288				
Standard Deviation, SD					0.5037	
R^2					0.9004	
Adj. R^2					0.8008	

[a] For coded variables.

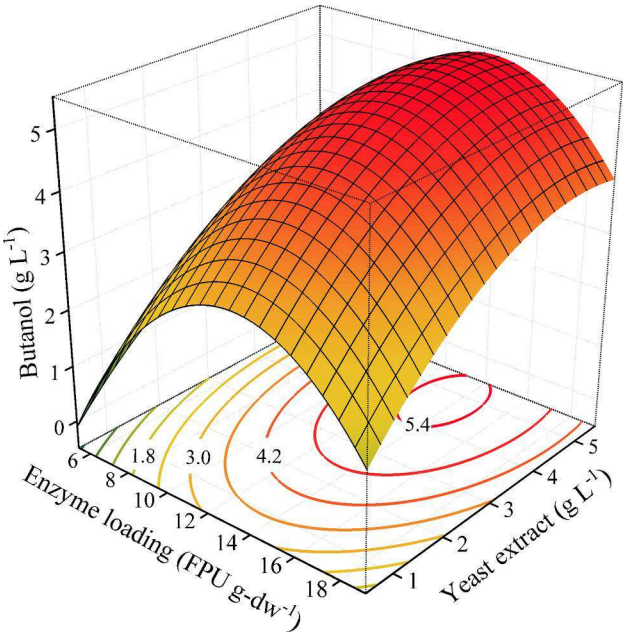

Fig. 2. The response surface and the corresponding contour plot for butanol production (g L^{-1}) at 48 h in the SSF process: combined effect of enzyme loading (FPU g-dw^{-1}) and yeast extract concentration (g L^{-1}). Initial pH = 6.4.

total concentration of sugars in the fermentation broth was less than 1.5 g L^{-1} and the butanol (ABE) yield resulted in 0.217 g g^{-1} (0.341 g g^{-1}) considering the maximum sugar concentration released in the two control experiments. It should be noted that both glucose and xylose were nearly completely consumed by the microorganisms, thus, maximum utilisation of the sugars released in the saccharification step was reached. Guan et al. [44] also pointed out that the SHF process showed higher ABE yields than those obtained in the SSF process from fermenting Kraft paper mill sludge by *C. acetobutylicum* ATCC 824.

The most remarkable difference between the one-step and two-step processes was found in the overall butanol productivity. A productivity of 0.040 g $L^{-1}h^{-1}$ was achieved in the SHF process, while a value 2.7-fold higher (0.109 g $L^{-1}h^{-1}$) was reached in the SSF process. This greater productivity is related not so much to the increase (by 8%) in the final butanol concentration but instead to the lower operation time needed to carry out the valorisation process of the RS. The SHF process needed a total of 120 h (72 h of enzymatic hydrolysis followed by 48 h of fermentation), while in the SSF process only 48 h were required to complete the butanol production at the same or even slightly higher levels than in the two-step process. Furthermore, the SSF process showed greater exploitation of the RS with a higher butanol-biomass ratio. Other authors compared the simultaneous process to the conventional SHF in the production of butanol from wheat straw [8,12], showing that SSF was more efficient and time-saving than SHF. Our results corroborated previous findings, revealing the potential of SSF to be less expensive than SHF in butanol production from the hydrolysate of straw. The greater efficiency of the SSF process could imply a reduction in equipment investment (only one vessel is necessary) and operational costs (lower production times, less contamination risk) in the production of butanol from RS.

3.3. Optimization of butanol production by SSF

Based on the above results, a RSM with full factorial CCD was performed for the SSF process to maximise butanol production by optimising three factors: enzyme loading, initial pH and yeast extract concentration. The model was validated by performing an experiment, with 3 replicates, at the optimum conditions.

3.3.1. Response surface methodology

The response surface methodology approach consisted of a five-level, three-factor CCD (Table 1) and subsequent linear regression analysis to fit the experimental data with a second-order model. Three independent variables were selected for the determination of the main effects and their interactions on butanol production. Enzyme loading (Z_1) and initial pH (Z_2), were found to be significant in the fractional factorial design of the SSF process, whereas yeast extract concentration (Z_3) was included for further study by enlarging its variation range. Based on the previous results, $FeSO_4{\cdot}7H_2O$ concentration was set to 0.02 g L^{-1}. Table 4 shows the CCD experimental matrix with variables in real terms and the observed and predicted values of butanol concentration after 48 h obtained from each condition. A total of 20 experimental runs were carried out, including 6 central point replications to check the experimental variability. The experimental results showed that the one-step process succeeded in producing butanol after 48 h within the ranges of the independent variables, achieving butanol concentrations from 1.06 to 5.31 g L^{-1}. Data of run 15 was not included

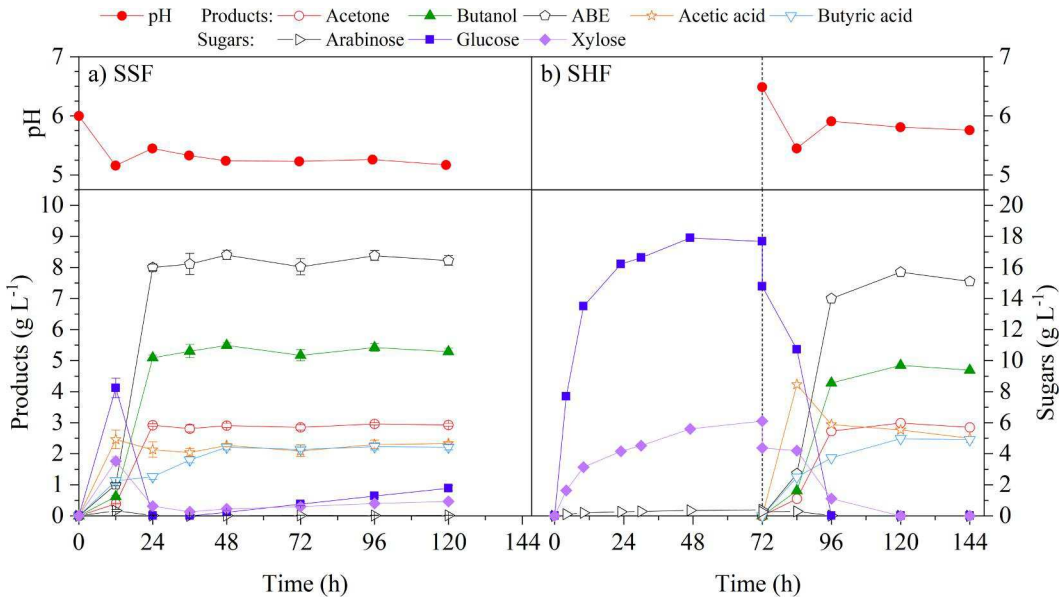

Fig. 3. Comparison of SSF and SHF processes. (a) SSF: CCD model validation at the predicted optimum conditions. Standard bar errors from three replicates; (b) SHF: Best results achieved, single run 7 of the 2^{4-1} fractional factorial design.

due to oxygen contamination detected by a resazurin indicator. The greater butanol production (5.31 g L^{-1}) was obtained in run 14 with an ABE concentration of 8.48 g L^{-1} and a butanol:acetone mass ratio of 1.7:1 (ethanol was not detected). Furthermore, a butanol productivity of 0.111 g $L^{-1}h^{-1}$ was reached with a butanol yield of 0.298 g g of consumed $sugar^{-1}$ and a butanol-biomass ratio of 48.8 g per kg of raw RS (ABE-biomass ratio of 78.0 g per kg of raw RS). The second-order model obtained for the concentration of butanol (g L^{-1}) in terms of actual factors was as follows:

$$\text{Butanol concentration} = -41.9 + 1.018Z_1 + 10.69Z_2 + 3.26Z_3 - 0.0641Z_1^2 - 0.804Z_2^2 - 0.1304Z_3^2 + 0.0783Z_1Z_2 + 0.0039Z_1Z_3 - 0.324Z_2Z_3 \quad (4)$$

The analysis of variance (ANOVA) and coded regression coefficients of the second-order model for butanol production are presented in Table 5. The model was highly significant at the 95% significance level, with a p-value of 0.0015, whereas the lack-of-fit was not significant (p-value of 0.0500). The low standard deviation (SD) value of 0.5037 g L^{-1}, measured in the units of the response variable indicates that the data values are not far from the fitted values. The coefficient of determination (R^2) value was 0.9004, showing a good correlation between the experimental results and the predicted values, in which only 9.96% of the total variations were not explained by the model. The goodness of the predictions was also confirmed by the adjusted coefficient of determination (Adj. R^2: 0.8008), suggesting that this model could properly predict the effect of enzyme loading, initial pH and yeast extract on butanol production after 48 h from RS by SSF. As can be seen from the ANOVA of the model, only the linear coefficients of enzyme loading (Z_1) and yeast extract concentration (Z_3) were found to be significant (p-value of 0.0037 and 0.0009, respectively), whereas initial pH was not significant (p-value of 0.3531). Unlike in other SSF processes [45], the variation of the initial pH in the range studied was not crucial on the response, because the effect of this factor depends on the strain, raw material and type of pretreatment [38]. The coded coefficients of the significant linear effects showed the degree of importance of the factors on the response: yeast extract (0.6671) > enzyme (0.5290). The p-value of the quadratic effect of enzyme (Z_1Z_1) was 0.0002, indicating that this variable had the greatest effect on butanol production. The rest of quadratic and two-way interaction effects were found to be not significant.

3.3.2. Conjugated effect of enzyme and yeast extract

The response surface plot of the final model equation is shown in Fig. 2, where the combined effect of enzyme loading and yeast extract on butanol production at a constant initial pH of 6.4 (central point in the CCD) is presented. In this figure, the three-dimensional surface and the two-dimensional contours for the butanol concentration after 48 h are plotted. The surface plot shape shows the great effect of the enzyme loading in comparison with the effect of yeast extract concentration. In addition, the rounded shape of the contour plots reflects, besides ANOVA outcomes, that the interaction effect between both factors was weak. As it can be seen, there is a maximum on the butanol concentration within the range of the variables established in the experimental design. According to the second-order model, the optimal conditions of the significant factors needed to achieve a butanol concentration of 5.43 g L^{-1} were an enzyme loading of 13.5 FPU g-dw^{-1} and a yeast extract concentration of 4.7 g L^{-1}. It should be noted that an enzyme loading higher than 16.3 FPU g-dw^{-1} caused a sudden decrease in the butanol concentration. In the one-step process, apart from increasing the operational costs, a large enzyme load could be counterproductive by inhibiting bacterial growth, as other authors have already pointed out [45]. Yeast is essential for ABE fermentation from bacteria such as *C. acetobutylicum* DSM 792, unlike other sources of nitrogen such as NH_4Cl and $NaNO_3$ [46]. Bacteria use nitrogen in the formation of nucleic acids, proteins and cell wall components [47], so the increase in yeast extract concentration is usually related to the improvement of growth, which would lead to an increase in sugar consumption and a greater butanol production [48]. However, Al-Shorgani et al. [49] observed that an excessive reduction of the C/N ratio inhibits butanol production despite favouring the growth of *C. acetobutylicum* YM1.

3.3.3. SSF model validation

The validation of the predicted optimal conditions from the CCD results was carried out in three replicates by using an enzyme loading of 13.5 FPU g-dw^{-1}, a yeast extract concentration of 4.7 g L^{-1} and an initial pH of 6.4. The variation with time of the solvent concentration (acetone and butanol; ethanol was not detected), acid concentration (acetic and butyric acid), sugar concentration (glucose, xylose and arabinose) and pH are plotted in Fig. 3a. Butanol concentration at 48 h (5.49 ± 0.09 g L^{-1}) only differed by 1.09% from the value estimated from the model (5.43 g L^{-1}), suggesting the goodness of model fit to predict the butanol

Table 6
Comparison of ABE fermentation through SHF and SSF processes from different feedstocks.

Substrate	Pretreatment	Fermentation method	Pretreated biomass loading	Enzyme loading	Microorganism	Butanol (ABE) production (g L^{-1})	Butanol (ABE) yield (g g^{-1})	Butanol (ABE)-biomass ratio (g kg RS^{-1})	Butanol (ABE) Productivity (g $L^{-1}h^{-1}$)	Reference
Brewer's spent grain	Dilute acid hydrolysis	SHF	10% (w/w)	Celluclast 1.5L (15 FPU g-dw^{-1}), Novozyme 188 (15 IU g-dw^{-1})	*C. beijerinckii* DSM 6422	6.1 (8.2)	0.20 (0.26)	28 (38)	0.06 (0.08)**	[50]
Rice straw	Ethanol organosolv	SHF	8% (w/w)	Celluclast 1.5L (25 FPU g-dw^{-1}), Novozyme 188 (40 IU g-dw^{-1})	*C. acetobutylicum* NRRL B-591	7.1 (10.5)	–	70 (103)	0.10 (0.15)**	[33]
Paper sludge	None	SSF	5% (w/v)	Cellic CTec2 (15 FPU g $glucan^{-1}$)	*C. acetobutylicum* ATCC 824	8.5 (14.5)	0.18 (0.30)	92 (157)	0.07 (0.12)	[44]
Oil palm empty fruit bunch	Alkaline	SSF	5% (w/v)	Acremonium cellulase (15 FPU g-dw^{-1})	*C. acetobutylicum* ATCC 824	4.0 (7.4)	0.16 (0.30)	80 (148)*	0.03 (0.06)	[45]
Wheat straw	Ammonium sulfite	SSF	9% (w/v)	Cellulase (5 FPU g-dw^{-1}), Xylanase (10 IU g-dw^{-1})	*C. acetobutylicum* ATCC 824	12.6 (19.8)	–	110 (173)	0.09 (0.14)	[12]
Rice straw	Microwave assisted hydrothermolysis	SSF	9% (w/v)	Cellic CTec2 (12 FPU g $glucan^{-1}$)	*C. beijerinckii* DSM 6422	5.5 (8.4)	0.31 (0.47)	51 (77)	0.11 (0.18)	This study

*The butanol and ABE-biomass ratio was calculated considering that the solid recovery was 100%.
**The butanol and ABE productivity was calculated without considering the enzymatic hydrolysis time.

concentration. Butanol yield and productivity were obtained as 0.306 ± 0.004 g g of consumed $sugar^{-1}$ and 0.114 ± 0.002 g $L^{-1}h^{-1}$ respectively. No increase in butanol concentration was observed after 48 h. Interestingly, 93% of the maximum value was already reached at 24 h, giving a productivity of 0.212 ± 0.004 g $L^{-1}h^{-1}$. The production of solvents resulted in 8.00 ± 0.10 g L^{-1} of total ABE at 24 h, when the concentration of acetone reaches its highest value (2.92 ± 0.04 g L^{-1}), and 8.40 ± 0.15 g L^{-1} at 48 h. Ethanol was not detected in significant concentrations throughout the study, which is positive for further downstream. Glucose and xylose accumulation were observed during the first 12 h, then decreased rapidly, indicating that enzyme hydrolysis was not the rate-limiting step unlike bacterial metabolism. This reversed after 48 h, when a slight increase in sugars was observed in the fermentation broth. For comparison purposes, the run 7 of the 2^{4-1} fractional factorial design of the SHF processes (highest butanol production for this configuration) was included in Fig. 3b. One of the main observable differences is that in order to achieve the maximum butanol production (4.85 g L^{-1}), 72 extra hours are required compared to the one-step process.

A comparison of the results of this study with those derived from the SHF and SSF processes reported in the literature is summarised in Table 6. Among the studies presented, different species of the genus *Clostridium* and different lignocellulosic substrates were used. When comparing butanol and ABE production, the achieved concentrations (5.5 and 8.4 g L^{-1}, respectively) were within the published values (4.0–12.6 g L^{-1} for butanol and 7.4–19.8 g L^{-1} for ABE), although they were in the lower range due to the low sugar concentration derived from the hydrolizated RS. The yield values of butanol and ABE found in this study (0.31 g g^{-1} and 0.47 g g^{-1}) were much higher than those achieved in the literature (0.16–0.20 g g^{-1} and 0.26–0.30 g g^{-1}), thus corroborating the notion that solvent production was limited not due to the capacity of the bacterial strain but rather to the limited release of sugars from the lignocellulosic material (22.98 g L^{-1}). This restriction is also indicated by the butanol (51 g kg RS^{-1}) and ABE-biomass ratio (77 g kg RS^{-1}); parameters reflecting the overall conversion from raw RS to solvents that need to be increased for a large-scale production. Besides the high yield, the productivity of butanol (0.11 g $L^{-1}h^{-1}$) and ABE solvents (0.18 g $L^{-1}h^{-1}$) was higher than that previously reported for the SSF process, where it takes between 72 and 144 h to reach the maximum concentration of butanol, unlike the 48 h required in our study. Compared with SHF from the literature, the values were even better, as reported productivities do not take into account the required time for the biomass saccharification (48–72 h more). This is of great interest, as high productivities are necessary to ensure an adequate butanol removal rate in *in-situ* product removal processes [51]. Further study is necessary in order to increase the release of sugars from the RS by enhancing the pretreatment method. The use of large concentrations of biomass can lead to problems such as inappropriate energy efficiency in microwave pretreatment [18], decrease of mass transfer [52] and decrease of substrate conversion due to enzymatic inhibition [53]. Therefore, investigations will be focus on improving the delignification and ash removal rather than to increase the biomass loading.

4. Conclusions

The serious environmental problems arising from the consumption of fossil fuels are increasing interest in producing biobutanol from lignocellulosic waste as a promising alternative energy source. In this study we demonstrated the feasibility of using hydrolysed rice straw by microwave irradiation as a substrate. By an adequate selection of operational conditions, fermentation time was reduced to 48 h with nearly total consumption not only of glucose, but also of xylose, resulting in high productivity which is a great advantage for scaling-up. Besides, the SSF process was shown to be a favourable configuration with the potential capability to reduce substantially the production cost when compared with a conventional SHF process. From these promising

results, further research on pretreatment conditions in order to improve the release of sugar concentrations from saccharification are of great interest to increase the butanol-biomass ratio prior to scale-up.

CRediT authorship contribution statement

Alejo Valles: Investigation, Formal analysis, Writing - original draft, Visualization. **F. Javier Álvarez-Hornos:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision. **Vicente Martínez-Soria:** Conceptualization, Methodology, Visualization. **Paula Marzal:** Conceptualization, Methodology, Resources. **Carmen Gabaldón:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fuel.2020.118831.

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Table S1. ANOVA of the 2^{4-1} fractional factorial design for the SHF and for the SSF processes.

Source	Significant	Sum of squares	Degrees of freedom	Mean square	*F* value	*p*-value Prob > F	Coefficient[a]
Statistics of regression for the SHF process							
Model	Yes	6.4374	6	1.0729	475.58	0.0351	
Yeast extract (g L^{-1})	No	0.0432	1	0.0432	19.14	0.1431	0.0735
$FeSO_4 \cdot 7H_2O$ (g L^{-1})	No	0.0186	1	0.0186	8.27	0.2131	0.0483
Initial pH	No	0.0200	1	0.0200	8.85	0.2064	-0.0500
Saccharification buffer	Yes	6.2990	1	6.2990	2792.12	0.0120	-0.8873
R^2						0.9996	
Adj. R^2						0.9975	
Statistics of regression for the SSF process							
Model	Yes	22.8461	6	3.8077	493.34	0.0344	
Yeast extract (g L^{-1})	No	0.9526	1	0.9526	123.42	0.0571	-0.3451
$FeSO_4 \cdot 7H_2O$ (g L^{-1})	No	0.5923	1	0.5923	76.74	0.0724	0.2721
Initial pH	Yes	11.5984	1	11.5984	1502.72	0.0164	1.2041
Enzyme (FPU g-dw^{-1})	Yes	4.0666	1	4.0666	526.88	0.0277	0.7130
R^2						0.9997	
Adj. R^2						0.9976	

[a] For coded variables.

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Optimization of alkali pretreatment to enhance rice straw conversion to butanol

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ABSTRACT

The use of rice straw (RS) was enhanced to produce biobutanol as biofuel, for which the NaOH pretreatment was optimized by considering the butanol-biomass ratio that quantify the mass balance efficiency of the three sequential stages of the process: pretreatment, enzymatic hydrolysis and fermentation by *Clostridium beijerinckii*. The optimum point (solid loading of 5% w/v with 0.75% w/v NaOH at 134 °C for 20 min) of the best cost-wise option yielded an enhanced biomass use of 77.6 g kg RS^{-1}. A maximum butanol titer of 10.1 g L^{-1} was reached after 72 h of fermentation with the complete uptake of glucose and nearly complete uptake of xylose. The NaOH concentration was the most influential parameter. The appropriate dosage to maximize fermentable sugars instead of the mass balance efficiency of the three stages underestimated the biomass use by 13%, showing the importance of correctly selecting the variable response during optimization.

1. Introduction

In 2020 the European Commission updated its First Circular Economy Action Plan launched in 2015 [1]. Among the actions derived from the EU's agenda for sustainable growth, the recast Renewable Energy Directive (EU) 2018/2001 laid down a target for advanced biofuels contributing 3.5% of the total transport sector energy. These biofuels must be produced from feedstocks like lignocellulosic biomass (bagasse, straw, or forestry waste), food cellulosic materials, animal manure or algae, among others. Rice straw (RS), mainly composed of cellulose and hemicellulose, is a promising feedstock with a global production in the range of 370–520 million tons per year [2]. Alcohols like methanol, ethanol, propanols, and butanols have been proposed as biofuel candidates. Although butanol has several advantages over shorter alcohols including higher volumetric energy content, it is less corrosive and is more compatible with conventional fuels [3].

ABE anaerobic fermentation is a sustainable method of producing biobutanol from rice straw. Clostridia species such as *Clostridium acetobutylicum* and *Clostridium beijerinckii* carry out ABE fermentation through biphasic metabolism: the organic acids produced in the acidogenic phase are re-assimilated later in the solventogenic phase to produce acetone, butanol and ethanol [4]. Although there are some shortcoming in batch fermentation (product inhibition, dead time periods required for medium preparation and sterilization) which result in lower butanol productivity, batch configuration has been conventionally selected for industrial ABE systems in Europe because of its easy operation and minimum control [5]. However, these Clostridia species are not able to hydrolyze lignocellulosic biomass efficiently [6], so that sugar monomers need to be obtained in an upstream pretreatment stage, followed by hydrolysis. The pretreatment alters the complex polymeric biomass structure by breaking the lignin seal, removing lignin and/or increasing its porosity [7]. The pretreatment should meet the following requirements: low energy demand and overall costs, efficient and rapid release of sugars in the subsequent hydrolysis, reducing carbohydrate degradation and avoiding the formation of inhibitory compounds (e.g., acids, furans and phenols). Concerning pretreatment of rice straw, several methods have been proposed, including: alkaline pretreatment [8–10], acid pretreatment [8,10,11], steam explosion [11], organosolv pretreatment [12] and the recent microwave-assisted hydrothermolysis [13]. Alkaline pretreatment with sodium hydroxide, ammonium hydroxide, potassium hydroxide or calcium hydroxide is effective in biomass with low lignin content and has other benefits such as low sugar degradation and a non-corrosive nature [14]. This chemical method disrupts and removes the lignin-carbohydrate structure and causes the biomass to swell, which reduces polymerization and cellulose crystallinity and increases the internal surface area. Among the different alkaline reagents, sodium hydroxide leads to a more efficient straw delignification [15], as well as other effects such as alteration of the

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silica layers and the cuticle wax, so that NaOH pretreatment could be proposed as the best method of increasing enzyme accessibility to cellulose in RS biomass [16–18]. Indeed, Moradi et al. [8] obtained a higher butanol production and overall conversion from RS pretreating with NaOH instead of H_2SO_4.

The operational conditions of the pretreatment affect the overall performance of butanol production, since they govern the susceptibility of the substrate to hydrolysis and the subsequent fermentation of the liberated sugars [19]. The most common approach for optimizing pretreatment conditions such as temperature, time, reagent concentration and solid loading focuses on evaluating their effect on the hydrolysis performance or on the delignification degree. For example in RS pretreatment, Singh et al. [20] used reducing sugar concentration as a response variable, Kim and Han [16] employed glucose recovery from untreated biomass and Hosseini et al. [15] and Mukherjee et al. [17] adopted the quantity of lignin removed from the biomass. Nevertheless, RS hydrolysates with high glucose concentration it can lead to reduced efficiency in terms of butanol production, butanol yield and overall butanol productivity [10].

Alternatively, the selection of a butanol to biomass ratio as response variable can be useful when pretreated hydrolysates are compared in terms of raw material conversion to butanol. The aim of this work was to optimize the alkaline pretreatment of RS considering the mass balance of the whole process from raw RS to butanol in order to assess the efficient use of lignocellulosic biomass. The global efficacy of the three serial steps: pretreatment, hydrolysis and ABE batch fermentation was defined by a single response variable. The effect of the temperature, time, NaOH concentration and solid loading was assessed on the (i) solid recovery, (ii) released sugars and (iii) produced butanol in order to integrate the efficiency of these three stages in the response variable, the butanol-biomass ratio (g butanol kg RS^{-1}). Pretreatment optimization was carried out by a preliminary fractional factorial design followed by a central composite design (CCD).

2. Materials and methods

2.1. Materials

RS was obtained from the Albufera Natural Park in Valencia (Spain). The raw material was milled and the particle size between 100 and 500 μm was selected by an ISO-3310.1 sieve (CISA, Spain). Before storing for further use, the biomass was dried in an oven at 45 °C to reduce residual moisture content below 5% w/w. The chemical composition (% dry weight basis) of the RS was: cellulose 35.8 ± 2.1%, hemicellulose 17.5 ± 1.4%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 14.3 ± 0.4%, ash 16.7 ± 0.1% and others 15.6 ± 3.7%.

Saccharification of the pretreated biomass was carried out by a commercial Cellic® CTec2 enzyme blend (Novozymes, Denmark). The cellulase activity resulted in a value of 157 filter paper units (FPU) mL^{-1} according to the National Renewable Energy Laboratory method [21]. *Clostridium beijerinckii* DSM 6422 (NRRL B-592), obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was stored at −80 °C in a Reinforced Clostridial Medium with 20% (v/v) glycerol. Before ABE fermentation the cells were grown following the procedure described elsewhere [13].

2.2. Alkaline pretreatment and enzymatic hydrolysis

Alkaline pretreatment was carried out in 500-mL glass bottles in which the RS was mixed with an NaOH solution (concentration ranging from 0 to 2% w/v) to achieve the appropriate solid loading (5 or 10% w/v), according to the experimental design described in Section 2.5. The bottles were then heated to 121 or 134 °C for a reaction time between 10 and 60 min in an autoclave (MED20, J.P. Selecta, Spain). The slurry was centrifuged at 4000 rpm for 6 min (Centrifuge 5804, Eppendorf, Germany), the solid phase was washed four times with deionized water and pH was adjusted to 6.5. Finally, the pretreated RS was dried at 45 °C for 24 h. The severity of the pretreatment conditions was estimated by the Severity Factor (SF) proposed by MacAskill et al. [22]:

$$SF = Log\left[time\ (min.) \times \exp\left[\frac{Temperature\ (^{\circ}C) - 100}{14.75}\right]\right] \quad (1)$$

Enzymatic hydrolysis was conducted in 100-mL conical flasks (with a working volume of 45 mL) containing 8% w/v of the pretreated RS to avoid end-product inhibition which was previously reported at higher RS loadings [9]. The commercial enzyme blend Cellic® CTec2 was added with a load of 15 FPU g-dw^{-1} based on the optimal configuration obtained in our previous study on simultaneous saccharification and fermentation of microwave-pretreated RS [13]. Hydrolysis was carried out at pH of 5.5 (50 mM acetate buffer), 50 °C and 200 rpm in an SI500 orbital shaker (Stuart, UK) for 72 h. After saccharification the samples were centrifuged at 4000 rpm for 10 min, filtered through 1.2 μm and stored at 4 °C until ABE fermentation.

2.3. ABE fermentation

Batch fermentations of 26 mL of the RS hydrolysate were performed in a 50 mL serum bottles with a working volume of 30 mL. The medium was composed of: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} NH_4Ac, 0.09 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.001 g L^{-1} $MnSO_4{\cdot}H_2O$, 0.02 g L^{-1} $FeSO_4{\cdot}7H_2O$ and 4 g L^{-1} of yeast extract. The initial pH was adjusted to 5.8, oxygen was displaced and bottles were autoclaved for 10 min at 121 °C. The bottles were inoculated with 2 mL (5% v/v) of *C. beijerinckii* DSM 6422, and incubated at 37 °C and 150 rpm for a maximum of 144 h.

2.4. Analytical methods

The efficiency of the pretreatment was characterized by analysis of sugars and inhibitory compounds from 1.5-mL samples of the RS hydrolysate. The chemical composition of the pretreated RS was determined for each pretreatment condition. Structural carbohydrates, lignin and ash were measured following the National Renewable Energy Laboratory procedures [23]. Fermentation was monitored by analyzing pH, cell growth, products of acids and solvents, and sugar consumption from 1-mL samples withdrawn every 24 h. The pH was measured by a Minitrode electrode (Hamilton, USA). Cell density (g-dw L^{-1}) was determined from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany) and using the linear correlation: g-dw L^{-1} = 0.2153·OD_{600} + 0.0689 (n = 10, R^2 = 0.9907).

Liquid samples were centrifuged at 10000 rpm for 5 min and filtered through 0.22 μm before HPLC analysis. Sugars (glucose, xylose and arabinose), acids (acetic acid, butyric acid and levulinic acid), solvents (butanol, acetone and ethanol) and other inhibitory compounds (furfural and 5-HMF) were analyzed by a liquid chromatograph (Agilent HPLC 1100 Series, Agilent Technologies, USA). An Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA) was operated at 50 °C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Sugars, butyric acid, butanol and ethanol were analyzed by a refractive index detector (RID). A diode array detector (DAD) was used to measure acetic, formic and levulinic acids at 210 nm, while acetone, furfural and 5-HMF were analyzed at 280 nm. The total phenolic compounds were determined by the Folin-Denis method [24], expressing phenolic concentration as gallic acid equivalents (GAE).

For the evaluation of the enzymatic hydrolysis, the glucose yield was defined as:

$$Glucose\ yield\ (\%) = \frac{Glucose\ released\ \left(g\ L^{-1}\right) \times 0.9 \times 100}{\left[80\ \left(g\ L^{-1}\right)/Solid\ recovery\ (\%)\right] \times Cellulose\ in\ raw\ RS(\%)} \quad (2)$$

The overall conversion of RS into biobutanol or ABE solvents was

calculated as follows:

$$Butanol\ (or\ ABE) \Big/ biomass\ ratio\ \left(g\ kg^{-1}\right) = \frac{Butanol\ (or\ ABE)\ produced\ (g) \Big/ V_{hydrolisate\ fermented}\ (L)}{\left[0.08\ \left(kg\ L^{-1}\right) \Big/ Solid\ recovery\ (\%)\right]} \times 100 \quad (3)$$

2.5. Design of experiments and statistical analysis

The alkaline pretreatment of RS was optimized in two subsequent statistical analyses. In both cases, the butanol-biomass ratio (g kg RS^{-1}), which indicates the mass balance efficiency of the three serial stages (pretreatment, hydrolysis and ABE fermentation) was selected as the response parameter. First, the significant variables were selected in the overall conversion to butanol by fractional factorial design. These variables were then optimized by the response surface method using CCD. Design of experiments and data analysis were conducted using the MINITAB® v.2020.1.0 commercial software (Minitab Inc., USA). Analysis of variance (ANOVA) was performed at a confidence level of 95% (p-value < 0.05).

2.5.1. Fractional factorial design and data analysis

The significant factors affecting the butanol-biomass ratio at 72 h were screened by a 2^{4-1} fractional factorial design (resolution IV, 8 experiment runs). Table 1 summarizes the coded and real values of the four variables. Pretreatment temperature (X_1) and solid loading (X_4) were established as the categorical variables, while pretreatment time (X_2) and NaOH concentration (X_3) were the range variables.

2.5.2. Central composite design and data analysis

Based on the results of the fractional factorial design, a response surface method with CCD (composed of 13 experiments with 5 central point replications) was used to determine the optimal combination of the significant variables (pretreatment time and NaOH concentration). The established range for each factor was as follows: pretreatment time (from 20 to 60 min) and NaOH concentration (from 0 to 1% w/v). Finally, a validation step was carried out by three replicates using the optimized conditions for RS use.

3. Results and discussion

3.1. Screening key factors on the overall conversion to butanol

The influence of the pretreatment variables (temperature, time, NaOH concentration and solid loading) was initially assessed for maximizing the release of the fermentable sugar and the butanol-biomass ratio. Table 2 summarizes the results of the fractional factorial design, including the solid recovery from pretreatment (%), sugars released after 72 h of enzymatic hydrolysis (g L^{-1}), butanol concentration (g L^{-1}), and butanol-biomass ratio (g kg untreated RS^{-1}) obtained from 72 h ABE fermentation. The alkaline pretreatment provided total solid recoveries ranging from 43.0 to 85.7%, with the highest values for the lowest NaOH concentration (runs 1–4) despite the SF.

The influence of temperature, time and solid loading seems to be negligible on the partial biomass solubilization and/or degradation. Fig. 1 shows the chemical composition (%) of raw and pretreated RS of the 8 runs. All pretreatment experiments with the highest NaOH concentration led to pretreated RS enriched in cellulose (>49%, runs 5–8), while a minimum time of 40 min was required to achieve some cellulose enrichment for the lowest NaOH concentration (40–42%, runs 3–4). The cellulose enrichment came mostly from the preferential removal of acid insoluble lignin rather than the hemicellulose solubilization, except for run 3, which hardly altered the biomass structure. The highest biomass loading of run 3 explains the lower release of sugars than in run 4 (4.9 vs. 33.4 g L^{-1}), these being unsuitable operational conditions for butanol production. High degrees of delignification (80.3–97.6%) were found in the experiments with the highest alkali concentration (2% w/v, runs 5–8), while moderate delignification (57.0%) was achieved in run 4 with the lowest alkali concentration (0.2% w/v).

The highest cellulose recovery (84.1%) was from the soft alkaline conditions (run 4), which positively influenced the subsequent hydrolysis and fermentation processes. Alkaline pretreatment is known to be able to remove lignin from RS by producing pores on its surface [17]. Other pretreatments such as acid or microwave-assisted hydrothermolysis resulted in lower delignification degrees, which could limit enzymatic sugar recovery to some extent. Moradi et al. [8] obtained a 27% delignification from acid pretreatment (50 °C, 30 min and 85% H_3PO_4) and Valles et al. [13] obtained 13.3% delignification by microwave-assisted hydrothermolysis at a ramp temperature from 100 °C to 200 °C for 40 min. The delignification achieved in this study was better than those obtained with other RS alkaline pretreatments. For example, Kim and Han [16] achieved less than 80% delignification working at a longer reaction time (60 min) and lower temperature (100 °C). Mild alkaline conditions can also achieve high delignification but they require a high NaOH concentration. Moradi et al. [8] reported 76% delignification working at 0 °C, 3 h and 12% w/v NaOH.

The sugars released after 72 h of enzymatic hydrolysis of the pretreated samples confirmed the efficiency of alkaline RS pretreatment. The three samples (runs 1–3) with delignification degrees less than 20% provided the lowest sugar concentrations (<18 g L^{-1}, Table 2), since lignin hinders cellulase access to cellulose fibers [16] and binds non-productively to cellulase [25]. The maximum sugar concentration was obtained in run 7, reaching a value of 65.3 g L^{-1} (50.5 g L^{-1} glucose, 13.5 g L^{-1} xylose and 1.3 g L^{-1} arabinose) although this experiment did not lead to the highest delignification. This could be attributed to the lower hemicellulose content of this sample (16.0%, Fig. 1), as hemicellulose can inhibit hydrolysis since acetyl groups from xylan cause steric hindrance of enzymes [26]. Nevertheless, these results showed that non-specific adsorption of the enzyme to the remained lignin do not play an adversely effect. The results of glucose yield (Fig. 1) showed that by increasing SF and reducing the solid loading the consumption of NaOH can be reduced to achieve similar values (runs 4 and 5 resulted in glucose yields ~40%). Hydrolysates were measured to quantify the potential inhibitory compounds. The analysis outcomes showed that the viability of the subsequent fermentation would not be negatively affected by the presence of inhibitory compounds. Levulinic acid and furfural were not detected in any of the samples and HMF concentration was negligible (<0.01 g L^{-1}). Total phenolic compound concentration ranged from 0.14 to 0.32 g L^{-1}, below the inhibitory level (0.71 g L^{-1}) for *C. beijerinckii* DSM 6422 [27]. The acetic acid in the hydrolysate was mainly formed during alkaline pretreatment by hydrolysis of the acetyl groups of hemicellulose [28]. The concentration of acetic acid ranged from 2.58 to 4.45 g L^{-1}, which could be beneficial for ABE fermentation as it has been reported that 3 g L^{-1} of acetic acid enhanced solvent production by *C. beijerinckii* DSM 6422 [29].

The hydrolysates were fermented by *C. beijerinckii* DSM 6422 to assess the efficiency of the whole process. As can be seen in Table 2, maximum concentration (10.6 g L^{-1}) was obtained for the maximum released sugars (65.3 g L^{-1}, run 7). In terms of sugar conversion to butanol, run 4 achieved similar values to run 7 (0.16 g butanol g released

Table 1
2^{4-1} fractional factorial design of 4 variables.

Independent variables		Coded and real values	
		Level −1	Level +1
X_1	Temperature (°C)[a]	121	134
X_2	Time (min)	10	40
X_3	NaOH concentration (% w/v)	0.2	2.0
X_4	Solid loading (% w/v)[a]	5	10

[a] Categorical.

Table 2
2^{4-1} fractional factorial design matrix along with the values of SF, solid recovery (%), released sugars after 72 h-enzymatic hydrolysis (g L^{-1}), butanol production (g L^{-1}) and butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h of ABE fermentation.

Run	Real values				SF	Solid recovery	Released sugars	Butanol	Butanol-biomass ratio
	X_1[a]	X_2[b]	X_3[c]	X_4[d]		(%)	(g L^{-1})	(g L^{-1})	(g kg untreated RS^{-1})
1	121	10	0.2	5	1.62	78.4	17.3	2.8	24.7
2	134	10	0.2	10	2.00	83.4	10.4	1.7	16.4
3	121	40	0.2	10	2.22	85.7	4.9	0.3	2.5
4	134	40	0.2	5	2.60	72.3	33.4	5.2	42.9
5	121	10	2	10	1.62	55.4	43.8	4.6	29.3
6	134	10	2	5	2.00	44.0	61.2	4.4	22.2
7	121	40	2	5	2.22	43.0	65.3	10.6	51.9
8	134	40	2	10	2.60	48.6	46.1	2.1	11.4

[a] X_1: temperature (°C).
[b] X_2: time (min).
[c] X_3: NaOH concentration (% w/v).
[d] X_4: solid loading (% w/v).

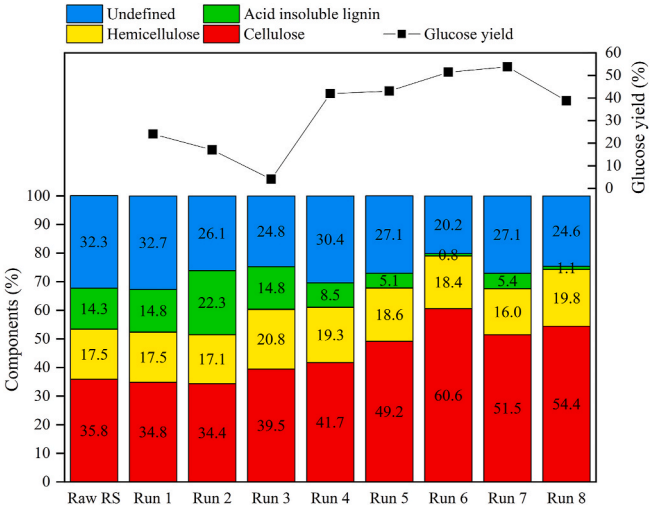

Fig. 1. Chemical composition of raw RS and RS after the different pretreatments included in the 2^{4-1} fractional factorial design with glucose yield (%, referred to untreated RS) after 72 h of enzymatic hydrolysis.

sugars^{-1}) in spite of its lower sugar concentration. The lower butanol production of runs 5, 6 and 8 was related to poor sugar conversion. After 24 h of fermentation, undissociated acids were higher in those samples than in run 7 (acetic and butyric acid: 23.0 ± 1.3 mM in runs 5, 6 and 8; 16.0 mM in run 7). Undissociated acids can pass across the *Clostridium* cell membrane, while their subsequent dissociation at the neutral internal pH of the cell can result in growth inhibition [4]. To keep the undissociated acid concentration at < 16 mM, initial pH was set at 5.8 for further experiments. RS-to-butanol conversion was analyzed to consider the loss of solids along with the sugar conversion (Table 2). The highest RS use was achieved in run 7 with a butanol-biomass ratio of 51.9 g kg untreated RS^{-1}, followed by run 4 with a value of 42.9 g kg untreated RS^{-1}. Both experiments had the lowest solid loading (5% w/v) but different NaOH requirements. Valles et al. [13] pretreated rice straw by microwave assisted hydrothermal hydrolysis, obtaining 51 g kg untreated RS^{-1} as the best value, showing that further optimization of alkaline pretreatment would be promising for efficient RS use.

The ANOVA regression model of the butanol-biomass ratio at 72 h (Table 2) was statistically significant with a p-value lower than 0.05 (95% confidence, p-value of 0.0479). Among the four variables included in the 2^{4-1} fractional factorial design, the solid loading (p-value of 0.0205) and the interaction between temperature and NaOH concentration (p-value of 0.0218) were found to be significant with a negative effect on the butanol-biomass ratio (g kg RS^{-1}). The good accuracy of the model was due to the coefficient of determination (R^2: 0.9805) and adjusted coefficient of determination (Adj. R^2: 0.9319) values. Considering the negative effect of the solid loading (categorical parameter) on the process efficiency the value of 5% w/v was selected for further optimization. For this solid loading, a model prediction was carried out to select the temperature level (categorical parameter) and the range of NaOH concentration and time to define the CCD. Fig. 2 shows the cube plot of fitted means of the butanol-biomass ratio, in which the vertices of the cube display the model results of the predicted values for all the combinations of the low and high levels of three parameters (temperature, NaOH concentration and time). As can be seen, there are two sets of temperature-NaOH conditions with RS-to-butanol conversion values higher than 40 g kg RS^{-1}. The two vertices lie on the plane intersecting the cube at 40 min corresponding to the pairs: 134 °C with 0.2% w/v NaOH and 121 °C with 2.0% w/v NaOH. Due to the potential saving in the reagent cost, it was decided to use 134 °C as the working temperature. From the edge at 40 min and 134 °C, it was decided to limit the maximum NaOH amount to 1% w/v.

3.2. Optimization of butanol-biomass ratio

Once the temperature and solid loading were set at 134 °C and 5% w/v from the results of the preliminary fractional factorial design experiment, a response surface method with full factorial CCD was carried out. The maximum butanol-biomass ratio was found from the best NaOH concentration and time conditions. To confirm the goodness of the optimization approach, the model was validated by running an experiment (3 replicates) in the optimum conditions.

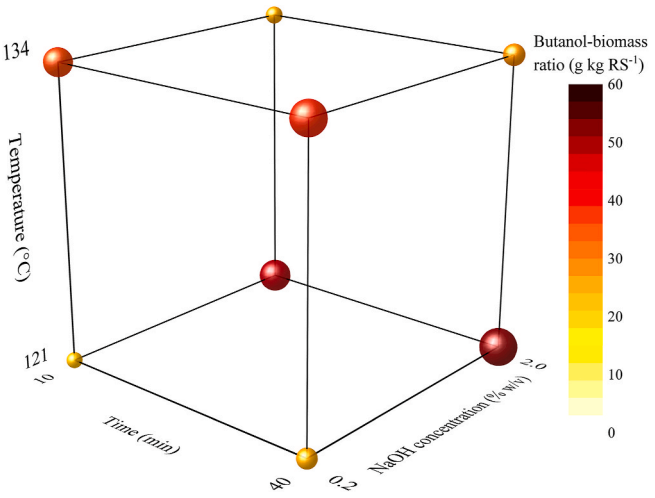

Fig. 2. 2^{4-1} fractional factorial cube plot using predicted model values of butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h. Solid loading = 5% w/v.

3.2.1. Response surface methodology

The RS-to-butanol conversion was maximized through a five-level, two-factor CCD, followed by linear regression analysis to adjust the experimental values to a second-order model. The experimental design is shown in Table 3 and includes the coded and real values of the range factors (NaOH concentration: Z_1; time: Z_2) for the 13 experimental runs, including five central point replications to assess the experimental variability. The central point conditions (0.5% w/v of NaOH concentration and 40 min pretreatment time) were selected from previous results. The axial point conditions ($\alpha = 1.4142$) were set to extend the NaOH concentration from 0 (hydrothermal equivalent) to 1% w/v with time in the range ± 20 min. As temperature was set at 134 °C, a narrower SF variation (2.30–2.78) was used than in the previous factorial design (1.62–2.60).

Table 3 also summarizes the response for the butanol-biomass ratio (g kg untreated RS^{-1}) along with the results of the three serial processes: (i) solid recovery (%) from alkaline pretreatment; (ii) released sugars (g L^{-1}) and glucose and xylose yields of untreated RS (%) in enzymatic hydrolysis; and (iii) butanol produced (g L^{-1}) after 72 h of ABE fermentation. Good reproducibility of the representative parameters was obtained for the three stages of the process for the central point replicates (run 9–13; solid recovery: 50.6 ± 0.0%; released sugars: 51.9 ± 1.3 g L^{-1}; butanol production: 8.8 ± 0.5 g L^{-1}). A broad solid recovery range was achieved from the experimental results (37.9–75.9%), which negatively correlated with the cellulose content of the samples. Of the two tested parameters, NaOH concentration had a strong effect, with RS pretreatment being less sensitive to time changes. This is in agreement with the results that Mukherjee et al. [17] obtained from alkaline pretreatment with a wider range of NaOH concentrations (0.73–12.73% w/v), time (39.55–140.45 min) and temperature (16.45–133.86 °C). They found that alkali concentration was more important in RS delignification during pretreatment than the other two factors. Especially when alkali concentration was as low as 0.15% w/v (runs 1, 3) or when alkali was not used (run 5) small solid losses were achieved, thus indicating the inefficiency of mild alkali usage for increasing the sugar released from RS. Indeed, these samples had a similar lignin content (13.2–16.9%) to the raw material. Insoluble lignin was extensively removed for 0.5% w/v NaOH or higher, while the content in the pretreated solid fractions varied from 0.7 to 6.4% (runs 2, 4, 6, 7–13). These findings show that similar RS delignification can be achieved by limiting NaOH concentration to a maximum of 1% w/v as by using 2% w/v NaOH (factorial fraction screening, Fig. 1).

All the pretreated solid fractions without any further detoxification were enzymatically hydrolyzed. As can be seen in Table 3, the concentration of the reducing sugars in the hydrolysate increased mainly with NaOH concentration, reaching the maximum value of 64.4 g L^{-1} (46.2 g L^{-1} glucose, 16.5 g L^{-1} xylose and 1.7 g L^{-1} arabinose) for the highest NaOH concentration (1% w/v, run 6), and the minimum value of 8.6 g L^{-1} (4.8 g L^{-1} glucose, 3.7 g L^{-1} xylose and 0.1 g L^{-1} arabinose) in the absence of NaOH (run 5). When low lignin removal was obtained (runs 1, 3 and 5), glucose yield varied from 11.6 to 31.7% and xylose yield varied from 20.7 to 43.7%. In the experiments with a high degree of delignification (runs 2, 4, 6, 7–13) more sugars were recovered from raw RS (53.3–59.7% for glucose and 46.4–56.8% for xylose). Although the experiments with high delignification lost more sugars, higher yields were obtained as enzymatic digestibility was improved. The glucose/xylose ratio in these experiments (2.6 ± 0.1) was substantially higher than the samples with NaOH concentration ≤0.15% w/v (1.6 ± 0.2). Solubilization of hemicellulose during pretreatment occurred at low NaOH doses. However, compared with the results of the fractional factorial screening (Table 1), it was confirmed that 1% w/v NaOH would be enough to reach the maximum concentration of fermentable sugars (~65 g L^{-1}).

The hydrolysates were subsequently fermented and the butanol production was evaluated. The low sugar content of run 5 made solvent production unfeasible as the bacteria metabolism did not have enough carbon sources to assimilate the acids produced during the acidogenic phase. In the rest of the samples, the ABE fermentation was not negatively impacted by the potentially inhibitory compounds produced during pretreatment. Levulinic acid and furfural were not detected in the hydrolysate and the concentrations of HMF (<0.01 g L^{-1}), while total phenolic compounds (0.31 ± 0.08 g L^{-1}) and acetic acid (3.95 ± 0.80 g L^{-1}) were below the inhibitory threshold level. Interestingly, the use of 0.15% w/v NaOH (runs 1 and 3) provided the highest butanol conversion of the released sugars (0.20 g butanol g released $sugars^{-1}$). Zhang et al. [30] reported that an acid insoluble lignin content of 8.55% in alkaline-pretreated corn stover by twin-screw extrusion released up to 0.74 g L^{-1} of soluble lignin compounds which inhibited ABE fermentation by *C. acetobutylicum* ATCC 824. In contrast, our results indicate that the lower lignin removal rate during RS pretreatment does not seem to have a negative impact on the Clostridia metabolism. The lack of by-product inhibition in our work could be attributed to the differences in the lignocellulosic biomass combined with the differences in the alkaline application. All the samples with a high lignin removal rate (runs 2, 4, 6–13) from the abundant carbon source in the hydrolysates (>50 g L^{-1}) produced high butanol concentrations (8.1–10.3 g L^{-1}), although the higher sugar concentration was not accompanied by higher sugar-to-butanol conversion. Although run 6 achieved the maximum

Table 3
CCD experimental matrix along with the values of solid recovery (%), released sugars (g L^{-1}) and glucose and xylose yield (%, referred to untreated RS) after 72 h-enzymatic hydrolysis, butanol production (g L^{-1}) at 72 h and the observed and predicted values of butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Run	Coded values		Real values		Solid recovery (%)	Released sugars (g L^{-1})	Glucose yield (%)	Xylose yield (%)	Butanol (g L^{-1})	Butanol-biomass ratio (g kg untreated RS^{-1})	
	Z_1[a]	Z_2[b]	Z_1[a]	Z_2[b]						Observed	Predicted
1	−1	−1	0.15	26	71.8	17.0	23.0	32.9	3.3	34.2	27.4
2	+1	−1	0.86	26	44.4	56.9	55.6	50.4	10.3	66.0	67.4
3	−1	+1	0.15	54	68.4	24.4	31.7	43.7	4.8	46.9	39.5
4	+1	+1	0.86	54	41.7	57.3	53.3	46.4	10.2	61.3	62.1
5	-α[c]	0	0.00	40	75.9	8.6	11.6	20.7	0.0	0.0	8.9
6	+α[c]	0	1.01	40	37.9	64.4	54.9	46.7	10.2	55.9	53.1
7	0	-α[c]	0.50	20	51.4	51.1	55.7	56.8	8.4	62.1	64.7
8	0	+α[c]	0.50	60	47.6	54.8	57.7	51.8	9.6	66.1	69.6
9	0	0	0.50	40	50.6	50.8	56.5	52.8	8.1	58.9	64.4
10	0	0	0.50	40	50.6	53.6	59.7	54.0	9.5	69.2	64.4
11	0	0	0.50	40	50.6	51.1	56.7	53.3	8.7	63.6	64.4
12	0	0	0.50	40	50.5	53.1	58.2	55.3	9.0	65.6	64.4
13	0	0	0.50	40	50.6	51.1	56.0	53.7	8.9	64.7	64.4

[a] Z_1: NaOH concentration (% w/v).
[b] Z_2: time (min).
[c] $\alpha = 1.4142$.

sugar concentration (64.4 g L^{-1}), the sugar-to-butanol conversion was the lowest (0.16 g butanol g released sugars^{-1}), due to the lower xylose uptake than in the samples with sugar concentrations in the range of 51–57 g L^{-1}. This could be attributed to the greater impact of the carbon catabolite repression phenomena for glucose levels of ~65 g L^{-1} [10]. These results show the importance of assessing the effectiveness of the pretreatment process by not only evaluating the amount of fermentable sugar released after hydrolysis but also the butanol conversion from fermentation.

The three-stage butanol-biomass ratio was therefore selected as the variable response for maximizing RS use. Regardless of the pretreatment reaction time, this ratio varied from 55.9 to 69.2 g kg untreated RS^{-1} when NaOH concentration was 0.5% w/v or higher (runs 2, 4, and 6–13), which is a considerable improvement on the previous fractional factorial screening (maximum value of 51.9 g kg untreated RS^{-1}, Table 1). The quadratic model obtained for the butanol-biomass ratio versus NaOH concentration (Z_1) and time (Z_2) is described by equation (4).

$$Butanol-biomass\ ratio = -2.9 + 212.8Z_1 - 0.00Z_2 - 132.5Z_1^2 + 0.0071Z_2^2 - 0.871Z_1Z_2 \quad (4)$$

The ANOVA and the coded regression coefficients of the quadratic model are summarized in Table 4. The results of the statistical analysis showed that the model was highly significant at the confidence levels (95%, p-value = 0.0004), whereas the lack-of-fit was not significant (p-value = 0.0777). The value of the coefficient of determination (R^2) was 0.9389, indicating that the experimental results had a good correlation with the predicted ones (Table 3), in which only 6.11% of the total variations were not explained by the fitted model. The value of the adjusted coefficient of determination (Adj. R^2: 0.8953) confirmed that equation (4) can adequately describe the effect of NaOH concentration and time on the butanol-biomass ratio obtained from RS after 72 h. The NaOH concentration had a much higher influence than time on the response. Both the linear (Z_1) and the quadratic (Z_1Z_1) coefficient were found to be significant with the same p-value (0.0002) but with opposite effects, while neither the linear (Z_2, p-value of 0.4584) or the quadratic (Z_2Z_2, p-value of 0.5725) coefficients of time were significant. The interaction effect between both factors was also found to be insignificant (Z_1Z_2, p-value of 0.2028).

The model was used to plot the three-dimensional response surface and the associated two-dimensional contour to map the optimal combination of the evaluated factors (Fig. 3a). The butanol-biomass ratio increases as NaOH concentration rises from 0 to 0.5% w/v, until reaching a saddle-shaped region. From 0.8% w/v NaOH, the response decreases as alkali concentration increases. The contour plot shows the weak interaction between NaOH concentration and time. Indeed, there are two opposing time values that led to the highest RS-to-butanol conversion: ~20–25 min or ~55–60 min for very similar NaOH concentrations (0.6–0.8% w/v). Of these two zones, the shorter time is the best cost-wise option, since the additional reagent is negligible relative to the extra time required. The model estimated the maximum butanol-biomass ratio at 72 h of fermentation (71.9 g kg untreated RS^{-1}) with 0.75% w/v NaOH and 20 min pretreatment time.

The combined effect of NaOH concentration and time on solid recovery after pretreatment (Fig. 3b), sugars released in enzymatic hydrolysis (Fig. 3c) and butanol produced in fermentation (Fig. 3d) were also plotted. Solid losses increase gradually with NaOH concentration due to the higher solubilization of the biomass in harsh alkaline conditions (Fig. 3b). The amount of fermentable sugar in the hydrolysate predicted to increase with NaOH concentration in the pretreatment rises to the optimal value (Fig. 3c), which was estimated by the model to be 61.7 g L^{-1} for an NaOH concentration of 0.88% w/v (and a pretreatment time of 39 min). As expected, both contour plots show very flat profiles versus time. The response surface plot for butanol production (Fig. 3d) shows a saddle shape similar to that of the butanol-biomass ratio (Fig. 3a), with two optimal regions near the minimum and maximum tested times ~20–23 min and ~55–60 min, but for higher NaOH dose (~0.9% w/v). Indeed, the butanol production quadratic model predicted a maximum butanol concentration of 10.6 g L^{-1} using 0.88% w/v NaOH and 21 min alkaline pretreatment. The discrepancies in alkali severity between butanol titer and RS-to-butanol conversion are attributed to the different extension of the solubilization of the structural components of the biomass during pretreatment. Other authors have also found that larger amounts of butanol and ABE can lead to lower mass balance efficiency [12,31]. Our results show the importance of selecting the right variable response during process optimization. The alkali dosage which maximizes fermentable sugars correlates well with the maximum butanol titer, but underestimates RS use by 13%. To improve the mass balance efficiency of ABE fermentation from the lignocellulosic biomass, the efficiency of all three pretreatment, saccarification and fermentation stages should be jointly considered when optimizing the operational parameters of the pretreatment process.

3.2.2. Model validation

The validation of the optimum value for the second-order model of the butanol-biomass ratio was carried out in triplicate using 5% w/v RS pretreated with 0.75% w/v NaOH at 134 °C for 20 min. The following profiles are depicted in Fig. 4: pH, cell density, sugar concentration

Table 4
ANOVA of the CCD model for butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Source	Degrees of freedom	Sum of squares	Mean square	*F* value	*p*-value Prob > F	Coefficient[a]
Model	5	4088.61	817.72	21.52	0.0004	
Linear	2	1985.18	992.59	26.12	0.0006	
Z_1: NaOH concentration	1	1961.79	1961.79	51.63	0.0002	15.66
Z_2: Time	1	23.39	23.39	0.62	0.4584	1.71
Square	2	2028.43	1014.21	26.69	0.0005	
Z_1Z_1	1	1938.68	1938.68	51.02	0.0002	−16.69
Z_2Z_2	1	13.32	13.32	0.35	0.5725	1.38
2-way interactions	1	75.00	75.00	1.97	0.2028	
Z_1Z_2	1	75.00	75.00	1.97	0.2028	−4.33
Error	7	265.98	38.00			
Lack-of-fit	3	209.72	69.91	4.97	0.0777	
Pure error	4	56.26	14.07			
Total	12	4354.58				
Standard Deviation, S					6.1642	
R^2					0.9389	
Adj. R^2					0.8953	

[a] For coded variables.

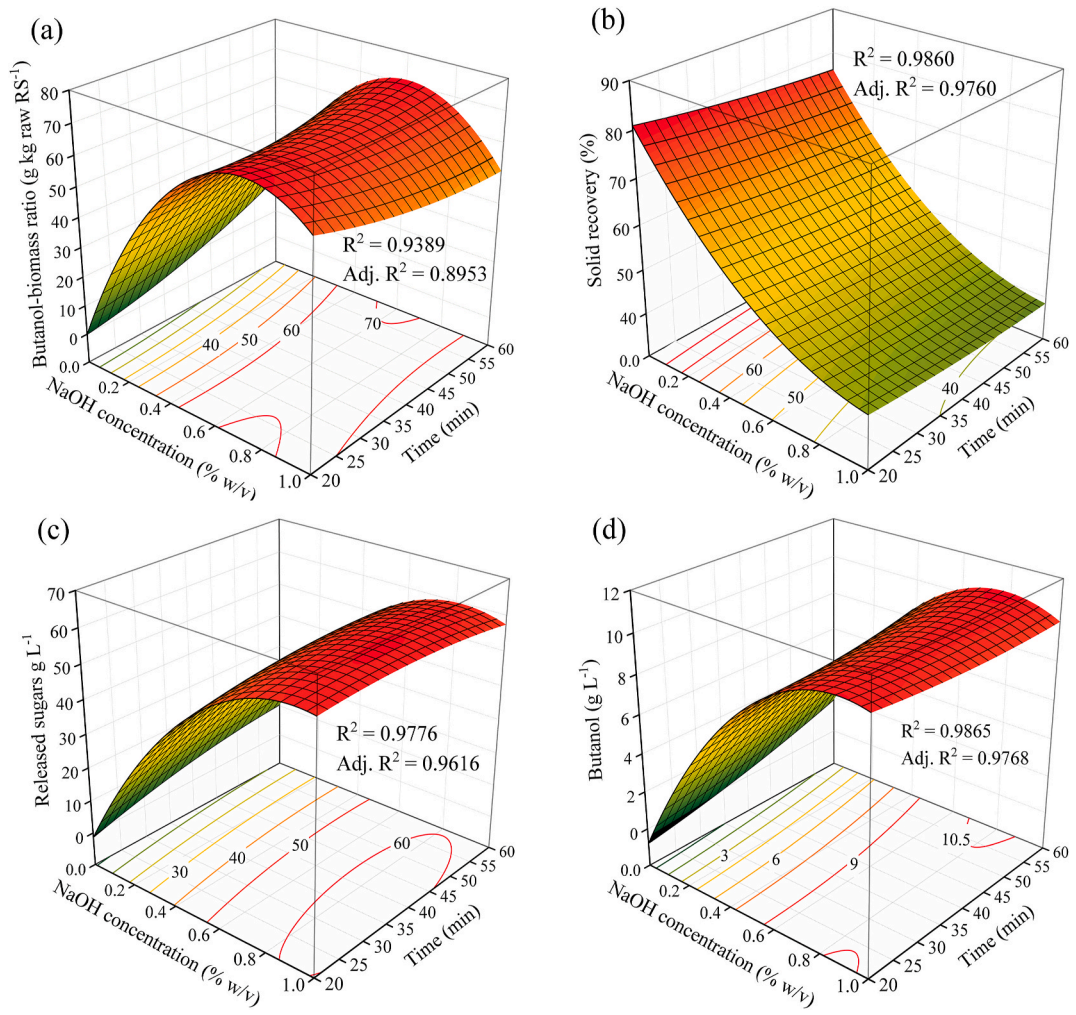

Fig. 3. Response surface and corresponding contour plot for (a) butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h, (b) solid recovery (%), (c) released sugars (g L^{-1}) after 72 h-enzymatic hydrolysis and (d) butanol production (g L^{-1}) at 72 h: combined effect of NaOH concentration (% w/v) and time (min).

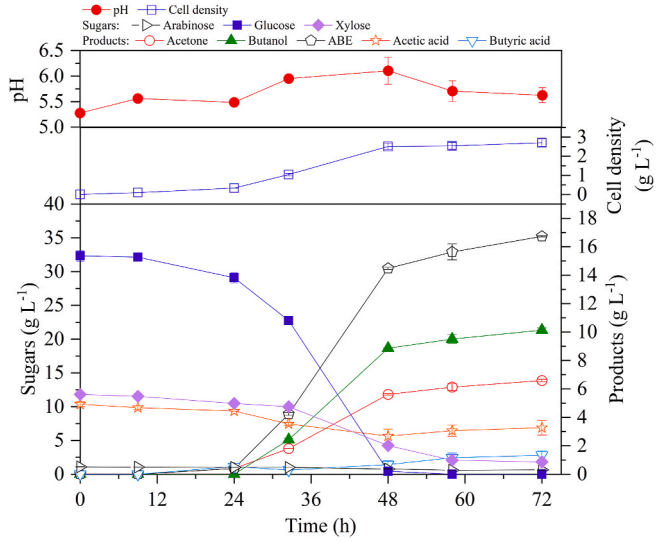

Fig. 4. CCD model validation at the predicted optimum conditions.

(glucose, xylose and arabinose), acid concentration (acetic and butyric acid) and solvent concentration (acetone and butanol, ethanol was not detected). A ~12 h lag phase was observed, after which the *Clostridium* culture started to grow by consuming glucose, indicating its adaptation to the nutritional environment. The exponential phase of cell growth was reached at ~24 h, although there was almost no acid accumulation or a notable change in pH shift in the first 24 h.

The absence of a substantial drop in pH during sugar consumption (acidogenesis stage) was related to the initial amount of acetic acid from the RS hydrolysate (4.9 ± 0.2 g L^{-1}). The acetic acid enhanced the early development of a mixed population of solvent and acidogenic cells with the coupled production of acids and solvents [32,33]. In this case, the early production of butanol and acetone as an acid consumer step caused a slight increase in pH from 24 to 33 h. Interestingly, 87% of the butanol (8.9 ± 0.1 g L^{-1}) and ABE solvents (14.5 ± 0.1 g L^{-1}, butanol:acetone mass ratio of 1.58) were produced in 48 h, accompanied by almost complete glucose depletion and about two-thirds of the xylose uptake, giving maximum butanol and ABE productivities of 0.185 ± 0.001 g L^{-1} h^{-1} and 0.302 ± 0.002 g L^{-1} h^{-1} respectively. Maximum butanol (10.1 ± 0.2 g L^{-1}) and ABE (16.7 ± 0.1 g L^{-1}, butanol:acetone mass ratio of 1.54) concentrations were obtained at the end of fermentation (72 h), with a butanol yield of 0.24 ± 0.01 g g^{-1} and ABE yield of 0.39 ± 0.01 g g^{-1}. In contrast to our results, production of similar butanol concentrations from non-detoxified NaOH-pretreated lignocellulosic biomass hydrolysates is related to an extensive fermentation time. For example, Cai et al. [34] achieved 9.4 g L^{-1} of butanol and 12.2 g L^{-1} of ABE from corn cob after 60 h of fermentation by *C. acetobutylicum* ABE 1301. Fernández-Delgado et al. [35] reported 11.3 g L^{-1} of butanol and 14.5 g L^{-1} of ABE after 96 h of fermentation from brewer's spent grain by

C. beijerinckii DSM 6422. Only Gao and Rehmann [36] achieved high solvent titers (12.3 g L^{-1} of butanol and 19.4 g L^{-1} of ABE) in short fermentation times (36 h) with NaOH-pretreated corn cob using *C. saccharobutylicum* DSM 13864. Our results indicate that RS alkaline pretreatment without further detoxification can achieve high butanol production accompanied by a high productivity. These results are promising for a further scale-up, as butanol productivity and not butanol concentration is the variable response of interest for in-situ butanol recovery processes [37]. In these integrated processes enhancing butanol productivity is critical to improving the butanol removal rate [38].

A butanol-biomass ratio of 77.6 ± 1.1 g kg untreated RS^{-1} was achieved at the end of fermentation with a solid recovery after pretreatment of 53.0 ± 0.1%. The observed value was slightly higher than the predicted one (71.9 g kg untreated RS^{-1}), validating the proposed model on butanol-biomass ratio. The discrepancy between the experimental and predicted values was 7.3%, which nearly matched the predicted deviation from the value of R^2 (6.1%), and was even better than that predicted from Adj. R^2 (adjusted coefficient of determination, 11.5%). Valles et al. [13] pretreated the same waste by microwaves and obtained a butanol-biomass ratio one-third lower (51 g kg untreated RS^{-1}) due to biomass delignification being less efficient than alkaline pretreatment. Few studies to date have assessed the overall lignocellulosic biomass conversion to butanol. Neither are there any previous reports on using the global mass balance efficiency of the three sequential steps: pretreatment, hydrolysis and fermentation, as the variable response in optimization. The RS converted to butanol achieved in the present study was quite similar to that obtained by Amiri et al. [12], 80 g butanol kg untreated RS^{-1} with an ethanol organosolv pretreatment, while a higher value (96 g butanol kg untreated RS^{-1}) was reported by Chi et al. [39] with NaOH-pretreatment. However, the high butanol-biomass ratios of these studies were not accompanied by high butanol titers (about 5–6 g L^{-1}). Our butanol production was nearly double and also achieved higher maximum productivity. It is important to note that the butanol stripping rate increases when the butanol titer in the reactor increases due to the enhanced butanol mass transfer associated with its higher driving force [40]. The promising mass balance efficiency, butanol titer and productivity achieved here with alkaline-pretreated RS shows promise for further research on reducing the cost by combining several stages in a single reactor (simultaneous saccharification and fermentation; simultaneous saccharification, fermentation and an *in-situ* recovery process).

4. Conclusions

Pretreating rice straw with NaOH was optimized to enhance rice straw conversion to butanol in batch ABE fermentation. Using the butanol-biomass ratio as the response variable has been identified as the key decision, since maximizing the sugar release without taking fermentation into account leads to underestimating RS use. By selecting the optimal conditions, the RS-to-butanol conversion was 77.6 g kg untreated RS^{-1} and a butanol titer as high as 10.1 g L^{-1} was achieved. In future studies, simultaneous saccharification and fermentation coupled with integrated recovery process will be carried out to increase the butanol productivity and final titer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2021.106131.

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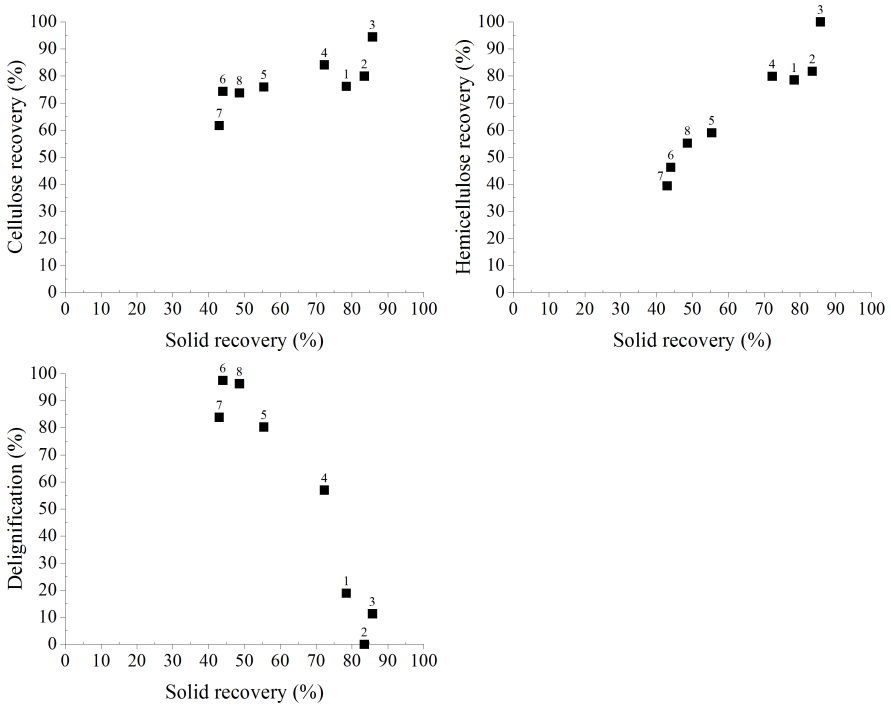

Figure S1. Cellulose and hemicellulose recovery (%) and delignification degree (%) versus solid recovery (%) obtained from the alkali pretreatment of the 2^{4-1} fractional factorial design experiment.

Table S1. ANOVA of the 2^{4-1} fractional factorial design model for butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Source	Degrees of freedom	Sum of squares	Mean square	*F* value	*p*-value Prob > F	Coefficient[a]
Model	5	1799.08	359.82	20.16	0.0479	
Linear	4	1006.46	251.61	14.09	0.0673	
X_1: Temperature (°C)	1	29.99	29.99	1.68	0.3243	-3.87
X_2: Time (min.)	1	32.28	32.28	1.81	0.3109	4.02
X_3: NaOH concentration (% w/v)	1	100.61	100.61	5.64	0.1409	7.09
X_4: Solid loading (% w/v)	1	843.58	843.58	47.26	0.0205	-20.54
2-way interactions	1	792.62	792.62	44.4	0.0218	
X_1X_3	1	792.62	792.62	44.4	0.0218	-19.91
Error	2	35.7	17.85			
Total	7	1834.78				
Standard Deviation, S					4.2251	
R^2					0.9805	
Adj. R^2					0.9319	

[a] For coded variables.

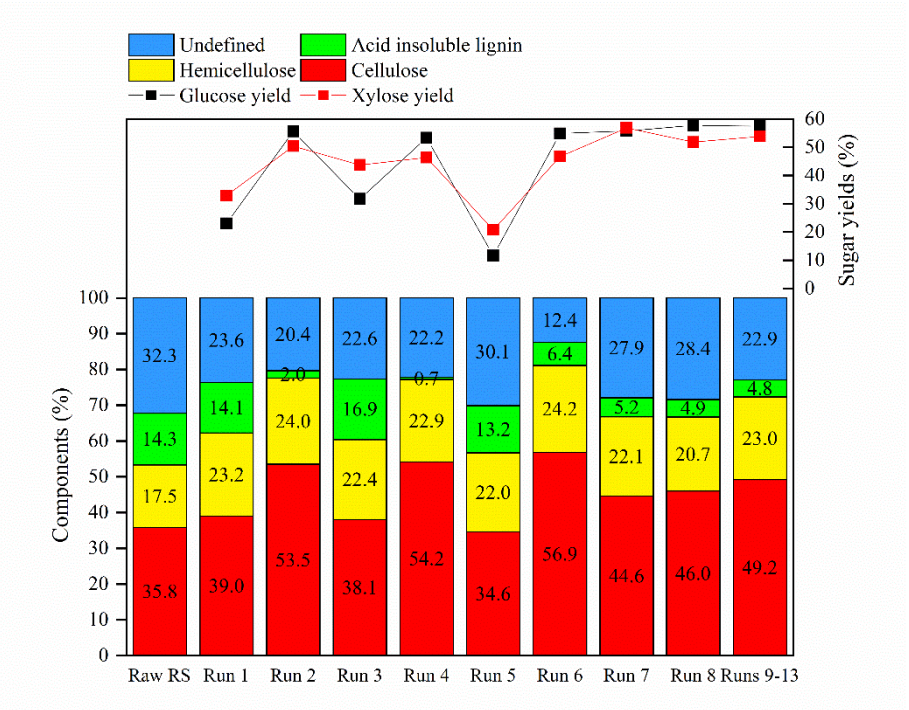

Figure S 2. Chemical composition of raw RS and RS after the different pretreatments included in the CCD with glucose and xylose yield (%, referred to untreated RS) after 72 h of enzymatic hydrolysis.

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Fed-batch simultaneous saccharification and fermentation including *in-situ* recovery for enhanced butanol production from rice straw

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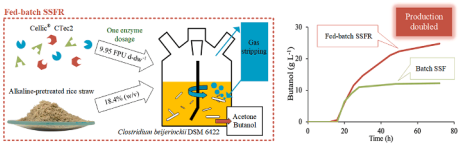
HIGHLIGHTS

- Fed-batch SSFR is an efficient configuration to produce butanol.
- A biomass load of 18.46% (w/v) was quickly processed to 24.80 g L^{-1} of butanol.
- High productivity of 0.344 g $L^{-1}h^{-1}$ was achieved.
- One enzyme dosage improved the economic viability of the process.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper describes a study of fed-batch SSFR (simultaneous saccharification, fermentation and recovery) for butanol production from alkaline-pretreated rice straw (RS) in a 2-L stirred tank reactor. The initial solid (9.2% w/v) and enzyme (19.9 FPU g-dw^{-1}) loadings were previously optimized by 50-mL batch SSF assays. Maximum butanol concentration of 24.80 g L^{-1} was obtained after three biomass feedings that doubled the RS load (18.4% w/v). Butanol productivity (0.344 g $L^{-1}h^{-1}$) also increased two-fold in comparison with batch SSF without recovery (0.170 g $L^{-1}h^{-1}$). Although fed-batch SSFR was able to operate with a single initial enzyme dosage, an extra dosage of nutrients was required with the biomass additions to achieve this high productivity. The study showed that SSFR can efficiently improve butanol production from a lignocellulosic biomass accompanied by the efficient use of the enzyme.

1. Introduction

Global warming, one of the greatest challenges facing world, is accelerating the transformation of the energy system. The current EU policy includes the production of biofuels from biomass wastes as one of the systems to achieve a successful climate-neutral transition (European Union: European Commission, 2020). Compared to other liquid biofuels such as ethanol, biobutanol has a higher energy density and is less hygroscopic and corrosive (Schubert, 2020). Butanol production by ABE fermentation from agricultural waste (rice straw, wheat straw or sugarcane bagasse among others) has been explored in the last decade (Abo et al., 2019; Vees et al., 2020). Although these lignocellulosic residues are an abundant and low-cost feedstock, they require pretreatment and saccharification prior to fermentation. In the case of rice straw (RS), which has a much higher ash and silica content than other agricultural lignocellulosic by-products (Satlewal et al., 2017), the pretreatment method should be selected considering that these components hinder accessibility to inner cellulose microfibers in enzymatic hydrolysis. Imman et al. (2015) reported the destruction of the RS silica layers after alkaline-catalyzed liquid hot water pretreatment with a 0.25% NaOH solution. Mukherjee et al. (2018) found that NaOH pretreatment reduced the percentage of silica by favoring delignification due to silica links with lignin. Together with the efficient removal of lignin and silica, the low degradation of sugars and its non-corrosive nature make alkaline

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pretreatment one of the most suitable methods to use on RS (Vivek et al., 2019). In a previous study, RS delignification by NaOH pretreatment has provided adequate saccharification, recovering ~ 60% of reducing sugars from the original carbohydrates (Valles et al., 2021).

Integrated bioprocessing, in which multiple processing steps are combined in a single operation, is an attractive approach for industrial-scale butanol production, for which simultaneous saccharification and fermentation (SSF) and fermentation with *in situ* product recovery (ISPR) are two of the most promising strategies (Ibrahim et al., 2018). In SSF, hydrolysis and fermentation take place together in the same vessel. This process could avoid the glucose inhibition of hydrolytic enzymes because sugars are simultaneously released and consumed by the bacteria. Also, SSF of microwave-pretreated RS has been shown to be more efficient than separate hydrolysis and fermentation (SHF) in terms of butanol production and productivity (Valles et al., 2020). ISPR reduces the high cost of downstream recovery processing, which is one of the major challenges in the commercialization of biobutanol (Abo et al., 2019). At the same time, it improves fermentation performance by alleviating butanol inhibition, which occurs at concentrations > 10 g L^{-1} (Ahlawat et al., 2019; Rochón et al., 2017). ISPR techniques include pervaporation, liquid extraction, gas stripping and perstraction. Of these, gas stripping is one of the simplest and most economic processes since it does not require either a membrane or chemicals and does not harm the culture (Li et al., 2020). By combining SSF with ISPR, the advanced SSFR (simultaneous saccharification, fermentation and recovery) configuration could markedly reduce the capital and operational costs of producing butanol from lignocellulosic biomass and food waste (Qureshi et al., 2020). Qureshi et al. (2006) carried out SSFR with *C. acetobutylicum* P260 from corn fiber arabinoxylan using gas stripping and reported that the full utilization of sugar and acids in SSFR, compared to SSF, increased ABE production (from 9.60 to 24.67 g L^{-1}) and productivity (from 0.20 to 0.47 g $L^{-1}h^{-1}$).

Combining SSFR with a fed-batch strategy, which allows large solid loadings without substrate or product inhibition is another method of drastically improving the cost-effectiveness of biobutanol production. Up to now, fed-batch has been considered to enhance ABE fermentation in SHF configurations (López-Linares et al., 2021; Rochón et al., 2017; Wen et al., 2018), but not in SSF, although fed-batch SSF was recently suggested as a promising alternative to be explored (Ibrahim et al., 2018). Feeding sterile substrate into the reactor is still a technical challenge to be overcome, although several studies on ethanol production by *Saccharomyces cerevisiae* have demonstrated the feasibility of feeding a sequential biomass to the reactor (Shengdong et al., 2006; Wang et al., 2013). For example, Shengdong et al. (2006) carried out fed-batch SSF in which an extra 10% (w/v) of pretreated RS was aseptically added in addition to a 10% (w/v) initial substrate concentration. This increased the reaction time by 144 h and achieved a much higher ethanol concentration (57.3 g L^{-1}) than that obtained in the single batch process (29.1 g L^{-1}). Fed-batch SSFR could thus solve some of the major challenges in ABE fermentation.

In the present work, a novel ABE fermentation approach was evaluated that consisted of fed-batch SSF with ISPR by gas stripping for butanol production from alkaline-pretreated RS. First, the effect of solid and enzyme loading on production was assessed in a batch SSF configuration using a central composite design (CCD). Based on the optimal values of solid and enzyme loading, fed-batch SSFR was then conducted with or without an additional enzyme dosage or medium compounds (buffer, yeast extract and minerals) in the subsequent feed cycles to further improve the economic viability of the process.

2. Materials and methods

2.1. Materials

RS from the Albufera Natural Park (Spain) was milled. The size fraction ranged from 100 to 500 μm was dried at 45 °C and stored. Its chemical composition (dry weight) was: glucan 35.6 ± 0.6%, xylan 17.6 ± 0.5%, arabinan 2.1 ± 0.2%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 10.4 ± 0.7%, ash 12.3 ± 0.7% and extractives 13.0 ± 1.3%. The Cellic® CTec2 commercial enzyme blend (Novozyme, Denmark) was used for enzymatic hydrolysis. A cellulase activity of 193 filter paper units (FPU) mL^{-1} was determined following the National Renewable Energy Laboratory (NREL) method (Adney and Baker, 1996) and additional information of the commercial enzyme blend can be found elsewhere (Aramrueang et al., 2017; dos Reis et al., 2013; Yang et al., 2017). *Clostridium beijerinckii* DSM 6422 (NRRL B-592) was purchased from DSMZ (Germany) and stored at −80 °C in a Reinforced Clostridial Medium (RCM) with 20% (v/v) glycerol. The pre-culture was statically grown for 24 h in 19 g L^{-1} RCM with 10 g L^{-1} glucose.

2.2. RS pretreatment

Alkaline pretreatment was conducted according to the protocol and the optimal conditions derived from a previous study (Valles et al., 2021). In brief, a solid loading of 5% (w/v) of RS was mixed with 0.75% (w/v) of NaOH solution and heated at 134 °C for 20 min. The solid fraction was then separated by centrifugation at 4000 rpm for 6 min (Mega Star 3.0, VWR, Germany) and washed several times with deionized water with a final pH adjustment to 6.5. The pretreated RS, which was previously dried at 45 °C for 48 h, was stored at −20 °C. After pretreatment a solid recovery of 48.19 ± 1.97% was obtained. The chemical composition of the alkaline-pretreated RS (dry weight) was: glucan 51.9 ± 0.6%, xylan 21.6 ± 0.3%, arabinan 3.8 ± 0.1%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 9.2 ± 0.5% and ash 7.4 ± 0.3%, indicating enriched carbohydrates with the removal of 57.5% acid insoluble lignin and 71.1% ashes.

2.3. Batch SSF

The SSF process was first optimized in 50-mL serum bottles in which solid (3.8 – 12.2% w/v) and enzyme loading (3.7 – 26.3 FPU g-dw^{-1}) were tested following the experimental design shown in Section 2.6. The medium (40 mL) contained: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} $C_2H_7NO_2$, 4 g L^{-1} of yeast extract, 0.09 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.001 g L^{-1} $MnSO_4{\cdot}H_2O$ and 0.02 g L^{-1} $FeSO_4{\cdot}7H_2O$. After oxygen displacement, the sealed bottles were autoclaved (121 °C for 10 min). Minerals (filter-sterilized by 0.22 μm) and enzyme were added before inoculation with 5% (v/v) of pre-culture. Incubation was conducted at 37 °C and 150 rpm for 72 h in an orbital shaker (SI500 model, Stuart, UK). Saccharification control without bacterial cells was conducted in triplicate with a solid loading of 8% (w/v) and an enzyme loading of 15 FPU g-dw^{-1}.

Once solid and enzyme loading were optimized, the process was scaled up in a 2-L stirred tank reactor (STR) using 500 mL of the above-mentioned medium with an RS loading of 9.2% (w/v). Start-up was similar to that performed with the serum bottles, but nitrogen was sparged after sterilization. After adding 4.73 mL of the enzyme blend (19.9 FPU g-dw^{-1}), the reactor was inoculated (5% v/v) and fermentation lasted 72 h at 37 °C and 120 rpm.

2.4. Fed-batch SSF coupled with in-situ gas stripping

The fed-batch SSF with ISPR by gas stripping took place in the 2-L STR. Fig. 1 shows a schematic diagram of the integrated reactor set-up. Gas stripping was performed by intermittently bubbling the fermentation gas (CO_2 and H_2) through the fermentation broth at 4 L min^{-1} by a vacuum gas pump (VP 86, VWR, Germany). The stripped solvents were recovered in a condenser at 4 °C with a cooling system (AD15R-30, VWR, USA). O_2-free distilled water was pumped by a peristaltic pump to keep constant the reactor volume at 500 mL. The condensate was periodically transferred for volume measurement and solvent analysis. The same start-up and operational conditions (37 °C and 120 rpm) described in Section 2.3 were used, ensuring an anaerobic

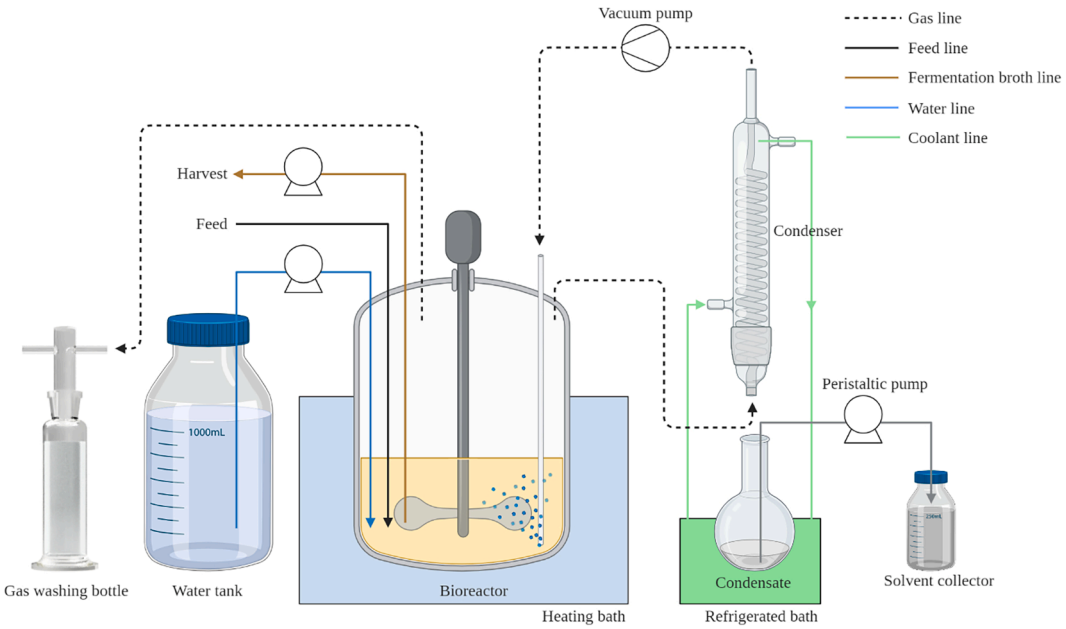

Fig. 1. Schematic diagram of fed-batch SSF with *in-situ* product recovery by gas stripping (SSFR). Created with BioRender.com.

environment by flushing the reactor headspace with nitrogen during feeding. Gas stripping started after 20 h and ended at 50 h. Dry alkaline-pretreated RS was added in three portions: 50% of the total quantity at the beginning of fermentation, 25% after 20 h and 25% after 30 h, thus doubling the solid loading from 9.2 to 18.4% (w/v). Three experiments were planned with different enzyme dosage and medium components together with the solid additions. In run 1, the enzyme blend Cellic® CTec2 was added to keep the enzyme loading constant at 19.9 FPU g-dw^{-1} throughout the entire process, while in runs 2 and 3 no extra enzyme was added to assess the Cellic® CTec2 cellulase activity over time. In runs 1 and 2, the fermentation was reinforced with medium components to keep the same ratio of buffer, yeast extract and minerals with feed solids, while in run 3 no additional medium components were used in order to assess the potential nutrient recycling from dead *C. beijerinckii* cells.

2.5. Analytical methods

The raw and pretreated RS were analyzed to determine the chemical composition according to NREL protocols (Sluiter et al., 2008). For all ABE fermentations, samples of 1 – 2 mL were periodically taken from the broth and, afterwards, were centrifuged (10000 rpm for 5 min) and filtered by 0.22-μm. A Minitrode electrode (Hamilton, USA) was used to pH measurements. The concentration of sugars (arabinose, glucose and xylose), inhibitory compounds (furfural, 5-HMF and levulinic acid) and fermentation products (acetone, butanol, ethanol, acetic acid and butyric acid) was determined by an Agilent HPLC (1100 Series, Agilent Technologies, USA) equipped with a refractive index detector, a diode array detector and an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA). The mobile phase (5 mM H_2SO_4) was set at 0.6 mL min^{-1}. The total phenolic compounds, expressed as gallic acid equivalents, was measured by the Folin-Denis method (Folin and Denis, 1912). A scanning electron microscope (SEM) S-4800 (Hitachi, Japan) was used to observe the *C. beijerinckii* DSM 6422 cells absorbed on the surface of the pretreated RS. Samples were coated with a gold and palladium mixture prior to imaging under SEM at an accelerating voltage of 20 kV.

2.6. Statistical design of experiments

Batch SSF was optimized by a CCD-based response surface method with concentration of butanol produced at 24 h (g L^{-1}) as the response variable in a total of 13 experiments with 5 central point replications. Solid loading (from 3.8 to 12.2% w/v) and enzyme loading (from 3.7 to 26.3 FPU g-dw^{-1}) were evaluated as the independent variables and their coded and real values are showed in Table 1. The statistical analysis was done on MINITAB® 19 software (Minitab Inc., USA). The optimal levels of the solid and enzyme loading predicted by the mathematical model were validated in triplicate. To obtain the product yield, a saccharification control was carried out in triplicate with a solid loading of 9.2% (w/v) and an enzyme loading of 19.9 FPU g-dw^{-1}.

3. Results and discussion

3.1. Batch SSF: Optimization

The optimum values of the solid and enzyme loading to maximize butanol production were assessed in batch SSF. For this, a five-level CCD was carried out using 50-mL serum bottles. The CCD experimental matrix with the real values of both independent variables is summarized in Table 2, along with the butanol production and sugar concentration in the culture broth at 24 h. The butanol titer at the end of fermentation (72 h) is also given along with the percentage of consumed sugars. These percentages were determined from the potential final sugar concentration estimated for each solid loading based on the sugar released by saccharification control (without inoculation).

At 24 h of fermentation, the butanol concentration ranged from 6.13 to 10.07 g L^{-1}, obtaining the minimum value from the lowest solid

Table 1
5-Level CCD of 2 independent variables. α = 1.4142.

Independent variables		Coded and real values				
		Level -α	Level −1	Central point (0)	Level + 1	Level + α
X_1	Solid loading (% w/v)	3.8	5.0	8.0	11.0	12.2
X_2	Enzyme loading (FPU g-dw^{-1})	3.7	7.0	15.0	23.0	26.3

Table 2
CCD experimental matrix along with the values of butanol production (g L^{-1}) at 24 and 72 h, remaining sugars (g L^{-1}, glucose, xylose and arabinose) at 24 h and consumed sugars (%, glucose and xylose) at 72 h.

Run	Real values[a]		24 h				72 h		
			Butanol	Sugars			Butanol	Consumed sugars	
			(g L^{-1})	(g L^{-1})			(g L^{-1})	(%)[b]	
	X_1	X_2		Glucose	Xylose	Arabinose		Glucose	Xylose
1	5.0	7.0	6.70	0.25	1.92	0.33	8.49	96.4	85.6
2	11.0	7.0	9.34	4.17	4.47	1.00	10.56	59.6	63.7
3	5.0	23.0	7.64	0.38	1.96	0.14	8.35	97.2	75.9
4	11.0	23.0	10.07	16.73	5.77	1.41	11.74	68.2	68.7
5	3.8	15.0	6.13	0.29	1.08	0.00	6.61	96.9	84.4
6	12.2	15.0	8.23	25.58	7.09	1.44	10.37	50.4	62.7
7	8.0	3.7	7.74	0.19	2.96	0.38	8.86	76.0	72.7
8	8.0	26.3	9.88	6.00	4.43	0.99	12.06	88.8	72.7
9 – 13	8.0	15.0	9.95 ± 0.35	1.33 ± 1.03	5.02 ± 0.18	1.04 ± 0.05	10.87 ± 0.46	94.2 ± 0.6	60.6 ± 2.8

[a] X_1: solid loading (% w/v); X_2: enzyme loading (FPU g-dw^{-1}).
[b] Final sugar concentration of saccharification control (solid loading of 8% w/v and an enzyme loading of 15 FPU g-dw^{-1}): 39.95 ± 0.23 g L^{-1} glucose, 13.35 ± 0.16 g L^{-1} xylose and 1.86 ± 0.00 g L^{-1} arabinose.

loading (3.8% w/v, run 5) and the maximum value from the solid loading of 11.0% (w/v, run 4). The central point replicates (run 9 – 13) show the low experimental variability of the parameters studied (butanol production: 9.95 ± 0.35 g L^{-1}; residual glucose: 1.33 ± 1.03 g L^{-1}, xylose: 5.02 ± 0.18 g L^{-1} and arabinose: 1.04 ± 0.05 g L^{-1}). After 72 h of fermentation, the maximum butanol concentration of 12.06 g L^{-1} was obtained with 8.0% (w/v, run 8), whereas higher solid loadings had a negative effect on the sugars converted to butanol with the consumption of reducing sugars below 70%. The negative impact of high biomass loading on ABE-SSF due to mass transfer limitations or the accumulation of inert components such as ashes, among other factors, was previously reported (Guan et al., 2016; Razali et al., 2018). In this work, the hydrolysis process seems not adversely impact by high solid loadings, due to the fact that a noticeable accumulation of glucose was observed for run 4 (16.73 g L^{-1}) and run 6 (25.58 g L^{-1}); both experiments corresponding to the combination of high solid loadings (≥11%) and high enzyme loadings (≥15 FPU g-dw^{-1}). Meanwhile, low acid concentrations were observed at 24 h for the whole set of experiments (acetic acid ranging from 0.95 to 1.38 g L^{-1}, butyric acid ranging from 0.60 to 1.89 g L^{-1}, total free acid concentration less than 6 mM) which were accompanied by butanol concentrations higher than 6 g L^{-1}. Thus, indicating a quick transition from acidogenesis to solventogenesis metabolism, being acidogenesis the limitation step at high solid loadings. These results corroborated the importance of the solid loading for an efficient biomass processing on simultaneous saccharification and fermentation.

Regarding the effect of enzyme dosing, increasing enzyme loading from 7.0 (run 1) to 23.0 FPU g-dw^{-1} (run 3) led to an improvement of 14% in butanol production at 24 h (from 6.70 to 7.64 g L^{-1}) when 5.0% (w/v) RS was used. Nevertheless, with the same degree of enzyme increase (7.0 to 23.0 FPU g-dw^{-1}) but with 11.0% (w/v) RS, 24-h butanol production slightly improved from 9.34 (run 2) to 10.07 g L^{-1} (run 4). At the end of the fermentation, butanol concentration in run 4 reached 11.74 g L^{-1} with around 68% of the glucose and xylose consumed, so that the combination of high solid and enzyme loading did not achieve the best use of the biomass. 10 g L^{-1} is most likely a sub-lethal concentration of butanol for *C. beijerinckii* DSM 6422 that slows down the consumption of the sugar released when a high enzyme loading is used. Although it was not observed in these experiments, several authors have found that large amounts of enzyme in SSF processes can reduce butanol production and productivity by sugar inhibition and cellulase stress (Dong et al., 2016; Razali et al., 2018). Interestingly, ~9 g L^{-1} butanol was produced at 72 h from 8.0% (w/v) of pretreated RS with only 3.7 FPU g-dw^{-1} (run 7), showing alkaline-pretreated RS could be saccharified with low enzyme consumption. The results obtained from 8.0% (w/v) of solid showed that the use of 15.0 FPU g-dw^{-1} (run 9 – 13) is enough to achieve the same butanol production (1% of difference) at 24 h when almost twice the amount of enzyme is used (26.3 FPU g-dw^{-1}, run 8). The differences between both enzyme doses at the end of the fermentation shows a 10% variation in butanol production. This evidence is extremely important since the economic viability of the process is guaranteed by not adding extensive amounts of enzyme.

The results of the integrated SSF (Table 2) show that at least 79% of the final butanol production was reached at 24 h, thus indicating the fast fermentation profile obtained. With the aim of maximizing butanol production prior to developing the fed-batch SSFR alternative, instead of 72 h, butanol production at 24 h was therefore selected as the response variable in the optimization. After fitting the experimental data by means of a linear regression analysis, the following second-order model was obtained:

$$Butanol\,production(24h) = -4.77 + 2.630X_1 + 0.307X_2 - 0.1413X_1^2 - 0.00718X_2^2 - 0.0023X_1X_2 \quad (1)$$

where X_1 is the solid loading (% w/v) and X_2 is the enzyme loading (FPU g-dw^{-1}). Table 3 shows the analysis of variance (ANOVA) and the coded regression coefficients of the above quadratic model. At a confidence level of 95%, while the model was significant (p-value = 0.0006), as the lack-of-fit was not significant (p-value = 0.1466), Eq (1) could accurately predict the effect of solid and enzyme loading on butanol production at 24 h. The good agreement between the observed and predicted data was indicated by the high values of the coefficient of determination (R^2: 0.9315) and the adjusted coefficient of determination (Adj. R^2: 0.8826). According to R^2, only ~ 7% of the total disparity was not explained by the model. Furthermore, a standard deviation of 0.4887 g L^{-1} denoted the small difference between the experimental and fitted data in terms of units of the response. As can be seen in Table 3, for both the solid (X_1) and the enzyme (X_2), the p-values of linear (X_1 = 0.0006, X_2 = 0.0113) and quadratic effects (X_1X_1 = 0.0002, X_2X_2 = 0.0422) were lower than 0.0500, so that all the effects of the two factors evaluated were found to be significant. No interaction was found between solid and enzyme loading (X_1X_2, p-value = 0.8310). The relative

Table 3
ANOVA of the CCD model for butanol production (g L^{-1}) at 24 h.

Source	Degrees of freedom	Sum of squares	Mean square	*F* value	*p*-value Prob > F	Coefficient[a]
Model	5	22.74	4.55	19.04	0.0006	
Linear	2	10.87	5.44	22.76	0.0009	
X_1: solid loading (% w/v)	1	8.10	8.10	33.90	0.0006	1.01
X_2: enzyme loading (FPU g-dw^{-1})	1	2.77	2.77	11.61	0.0113	0.59
Square	2	11.86	5.93	24.82	0.0007	
X_1X_1	1	11.25	11.25	47.09	0.0002	−1.27
X_2X_2	1	1.47	1.47	6.15	0.0422	−0.46
2-way interactions	1	0.01	0.01	0.05	0.8310	
X_1X_2	1	0.01	0.01	0.05	0.8310	−0.05
Error	7	1.67	0.24			
Lack-of-fit	3	1.18	0.39	3.18	0.1466	
Pure error	4	0.49	0.12			
Total	12	24.41				
Standard Deviation, S					0.4887	
R^2					0.9315	
Adj. R^2					0.8826	

[a] For coded variables.

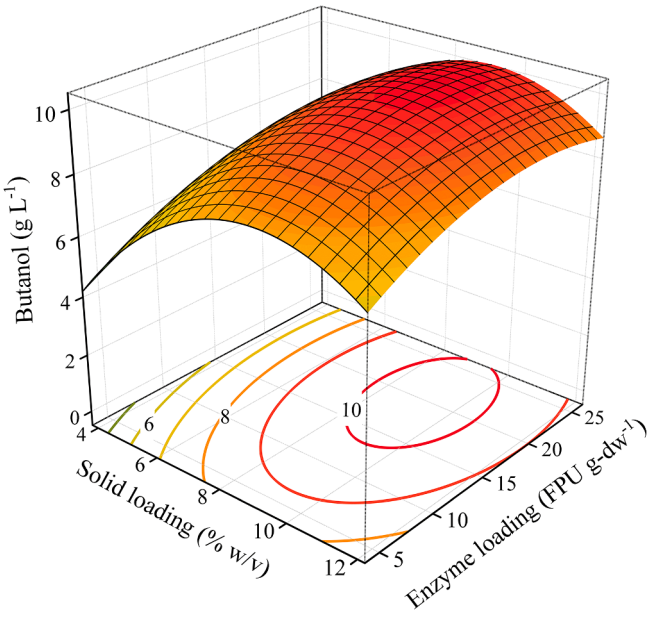

Fig. 2. Response surface and corresponding contour plot for butanol production (g L^{-1}) at 24 h: combined effect of solid loading (% w/v) and enzyme loading (FPU g-dw^{-1}).

importance of the variables, based on the coded coefficients, was as follows: solid loading ($X_1 = 1.01$, $X_1X_1 = -1.27$) > enzyme loading ($X_2 = 0.59$, $X_2X_2 = -0.46$).

The 3D response surface plot derived from the regression model and the corresponding 2D contour plot are shown in Fig. 2. According to the ANOVA results, these plots indicate that the variation of solids has a greater impact than that of the enzyme on the response and show the non-interaction between both factors. The maximum butanol production of 10.32 g L^{-1} at 24 h from 9.2% (w/v) solid and 19.9 FPU g-dw^{-1} enzyme loadings was estimated by the model. Butanol concentration rises to a peak value by increasing solid loading to 9.2% (w/v), since more fermentable sugars are released. From 9.2% to 12.2% RS (w/v), the response decreases because the metabolism of *C. beijerinckii* DSM 6422 seems to be adversely impacted by the buffering effect of some RS compounds such as ash. Regarding enzyme loading, Fig. 2 shows a flat area (~13 to 26 FPU g-dw^{-1}) around the optimum value in which varying enzyme loading would reduce butanol production by less than 3%, so that the model confirmed that neither sugar inhibition nor cellulase stress occurred.

3.2. Batch SSF: Model validation

The butanol production model at 24 h predicted by the CCD was validated through three identical assays performed on 50-mL serum bottles. Based on the optimal settings, 9.2% (w/v) alkaline-pretreated RS was hydrolyzed and fermented simultaneously by 19.9 FPU g-dw^{-1} of enzyme. Fig. 3a depicts the time fermentation profile of the products (acetone, butanol, acetic acid and butyric acid) and sugars (glucose, xylose and arabinose). Ethanol was not detected as it has been previously reported by others authors using strain *Clostridium beijerinckii* DSM 6422 (Plaza et al., 2017; Valles et al., 2021, Valles et al., 2020). At 12 h, before *Clostridium* metabolism was entirely active, 23.78 ± 4.67 g L^{-1} of sugars (17.74 ± 3.50 g L^{-1} glucose, 5.19 ± 1.02 g L^{-1} xylose and 0.85 ± 0.16 g L^{-1} arabinose) remained in the medium. From the subsequent uptake of monosaccharides, 9.27 ± 0.87 g L^{-1} of butanol and 15.52 ± 1.19 g L^{-1} of ABE (butanol:acetone mass ratio = 1.48) were produced at 24 h. A slightly lower production (~10%) was obtained by comparing the model's predicted response (10.32 g L^{-1}). This small discrepancy was due to a minor delay in fermentation, as it only takes 12 h to reach 10.67 g L^{-1} (Fig. 3a). When fermentation finished at 72 h, 11.89 ± 0.49 g L^{-1} of butanol and 19.42 ± 1.46 g L^{-1} of ABE (butanol:acetone mass ratio = 1.58) were obtained, thus giving a butanol productivity of 0.165 ± 0.007 g $L^{-1}h^{-1}$ and an ABE productivity of 0.270 ± 0.020 g $L^{-1}h^{-1}$. The butanol productivity increases to 0.386 ± 0.036 g $L^{-1}h^{-1}$ when considering 24 h, in which time 78% of the butanol had already been produced. The butanol-biomass ratio as parameter to assess the mass balance of the whole process from raw RS to butanol was calculated to be 62.6 ± 2.6 g-butanol kg-raw RS^{-1}. In the control assay conducted under optimal conditions but without inoculation, sugar concentration reached 60.00 ± 5.93 g L^{-1} (44.50 ± 2.19 g L^{-1} glucose, 13.36 ± 3.55 g L^{-1} xylose and 2.14 ± 0.19 g L^{-1} arabinose) at 72 h. From these values, butanol and ABE yields of 0.253 ± 0.020 and 0.412 ± 0.020 g g of consumed $sugar^{-1}$ were obtained. In comparison with those in the literature, the final butanol concentration was within the greatest values (10.2 – 13.0 g L^{-1}) reported for SSF systems from cellulosic material and different species of *Clostridium* (Dong et al., 2016; Guan et al., 2016; Qi et al., 2019). Whereas higher values of butanol-biomass ratio (80 – 110 g-butanol kg-raw RS^{-1}) have been reported for other cellulosic materials fermented in SSF systems (Guan et al., 2016; Qi et al., 2019; Razali et al., 2018), butanol-biomass ratio obtained in this study, 62.6 ± 2.6 g-butanol kg-raw RS^{-1}, improves the rice straw conversion to butanol in 23% from SSF process (Valles et al., 2020). Results are comparable not only in terms of butanol production but also in productivity to Dong et al. (2016), who achieved 13.0 g L^{-1} of butanol in 48 h using 9% (w/v) of alkaline-pretreated corn stover and *C. saccharobutylicum* DSM 13864. In contrast, SSF with *C. acetobutylicum* ATCC 824 (Guan et al., 2016; Qi

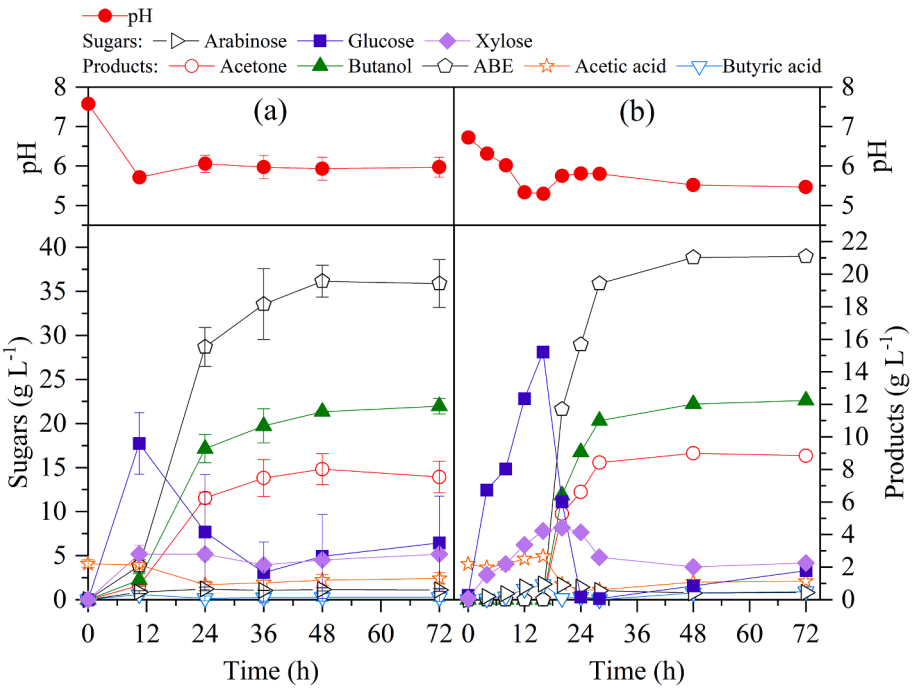

Fig. 3. CCD model validation. (a) Batch SSF in a 50-mL serum bottles. (b) Scale-up of the batch SSF to a 2-L reactor.

et al., 2019) showed a slower fermentation rate and low butanol production at 24 h (less than 4 g L^{-1}) and requiring between 120 and 144 h to obtain the above-mentioned final concentrations. *C. beijerinckii* DSM 6422 therefore seems to be a good candidate for SSF together with *C. saccharobutylicum* DSM 13864. In comparison with the previous results of *C. beijerinckii* DSM 6422 on SHF of hydrolyzates from 8% (w/v) of alkaline-pretreated RS (Valles et al., 2021), the overall butanol productivity (considering both hydrolysis and fermentation time) was 2.4 times higher in the one-step (SSF, 0.165 g $L^{-1}h^{-1}$) than two-step process (SHF, 0.070 g $L^{-1}h^{-1}$). This confirms that one of the SSF's great advantages over SHF is the time savings, which reduces operational costs, along with a reduced risk of glucose contamination and enzyme inhibition.

The optimized batch SSF was carried out in a 2-L STR with a working volume of 500 mL to evaluate the feasibility of the process in a bench-scale bioreactor. Scale-up was successful as no relevant differences were found between the sugar and product profiles of both experiments (Fig. 3). The butanol concentration at 24 h (9.06 g L^{-1}) and 72 h (12.24 g L^{-1}) in the 2-L reactor differed by less than 3% with respect to the values observed in the serum bottles, corresponding to a final conversion of raw RS to butanol of 64.4 g-butanol kg-raw RS^{-1}. The final production of ABE was 8% higher (21.09 g L^{-1}, butanol:acetone mass ratio = 1.38) and the productivity was 0.170 g $L^{-1}h^{-1}$ for butanol and 0.293 g $L^{-1}h^{-1}$ for ABE. At 72 h, 8.27 g L^{-1} of sugars (3.29 g L^{-1} glucose, 4.17 g L^{-1} xylose and 0.81 g L^{-1} arabinose) remained unused in the medium. Taking into account the sugar released in the control experiments without inoculation, 86% of the sugars were consumed, thus resulting in butanol and ABE yields of 0.237 and 0.408 g g of consumed $sugar^{-1}$, respectively. The maximum concentrations of total phenolic compounds (0.42 – 0.45 g L^{-1}) were just about half the inhibitory concentration (0.71 g L^{-1}) for *C. beijerinckii* DSM 6422 (López-Linares et al., 2019). Furfural, 5-HMF and levulinic acid were not detected and the maximum concentration of undissociated acids (acetic and butyric) was 12.51 mM, below the inhibitory level (16 mM, Valles et al., 2021). The end of butanol production and the observed increase in the concentration of residual sugars from 48 to 72 h thus suggests that fermentation was inhibited by butanol at a concentration of 12.24 g L^{-1}. Ahlawat et al. (2019) found 12.56 g L^{-1} as the threshold concentration of butanol, at which the growth of *C. acetobutylicum* MTCC 11,274 and sugar consumption stopped. The inhibitory concentration for *C. acetobutylicum* DSM 792 was 10.5 g L^{-1} (Rochón et al., 2017), showing that tolerance depends, among other factors, on the bacteria strain. To further improve RS butanol production and productivity, the optimized operational conditions (9.2% (w/v) of solid and 19.9 FPU g-dw^{-1} of enzyme loadings) were selected as the initial conditions of the fed-batch SSFR configuration.

3.3. Fed-batch SSF coupled with in-situ gas stripping.

A novel fed-batch SSF with ISPR by gas stripping was evaluated to assess the feasibility of using high amounts of biomass in ABE fermentation while avoiding both substrate and butanol inhibition. From an initial alkaline-pretreated RS loading of 9.2% (w/v), two additional biomass feedings were performed (as indicated by the arrows in Fig. 4) to avoid inefficient mixing or low water activity associated with the high amount of solids to be processed (18.4% w/v). The first additional feeding (25% of the whole) was done at 20 h, before expecting complete glucose depletion (Fig. 3b). This model helps to maintain biological activity once the solventogenesis has started. The second (25% of the whole) was carried out 10 h after the first, when solubilization of the previously added solid was ensured. Gas stripping was turned on at 20 h with the first biomass feeding to avoid butanol inhibition. The application time of gas striping was planned to keep the butanol concentration in the fermentation medium well below 12 g L^{-1}. It was turned off at 50 h when the concentration was less than 5 g L^{-1}. Three experimental runs were carried out to assess the recycling of enzyme and medium compounds (buffer, yeast extract and minerals) in the process.

The fermentation profile of run 1, where the final enzyme loading (19.9 FPU g-dw^{-1}) and the nutrient ratio over solids were the same as in the batch SSF, is depicted in Fig. 4a. As shown, the pH and sugar profile in the first 20 h was very similar to those of the batch SSF (Fig. 3b). The pH then remained stable at 5.74 ± 0.17 until the end of fermentation due to the re-assimilation of acids into solvents, with low concentrations of acetic acid (0.76 – 2.98 g L^{-1}) and butyric acid (0.00 – 0.40 g L^{-1}) since 20 h. The maximum observed concentration of undissociated acids (10.92 mM) was below the inhibitory value (16 mM). Regarding other

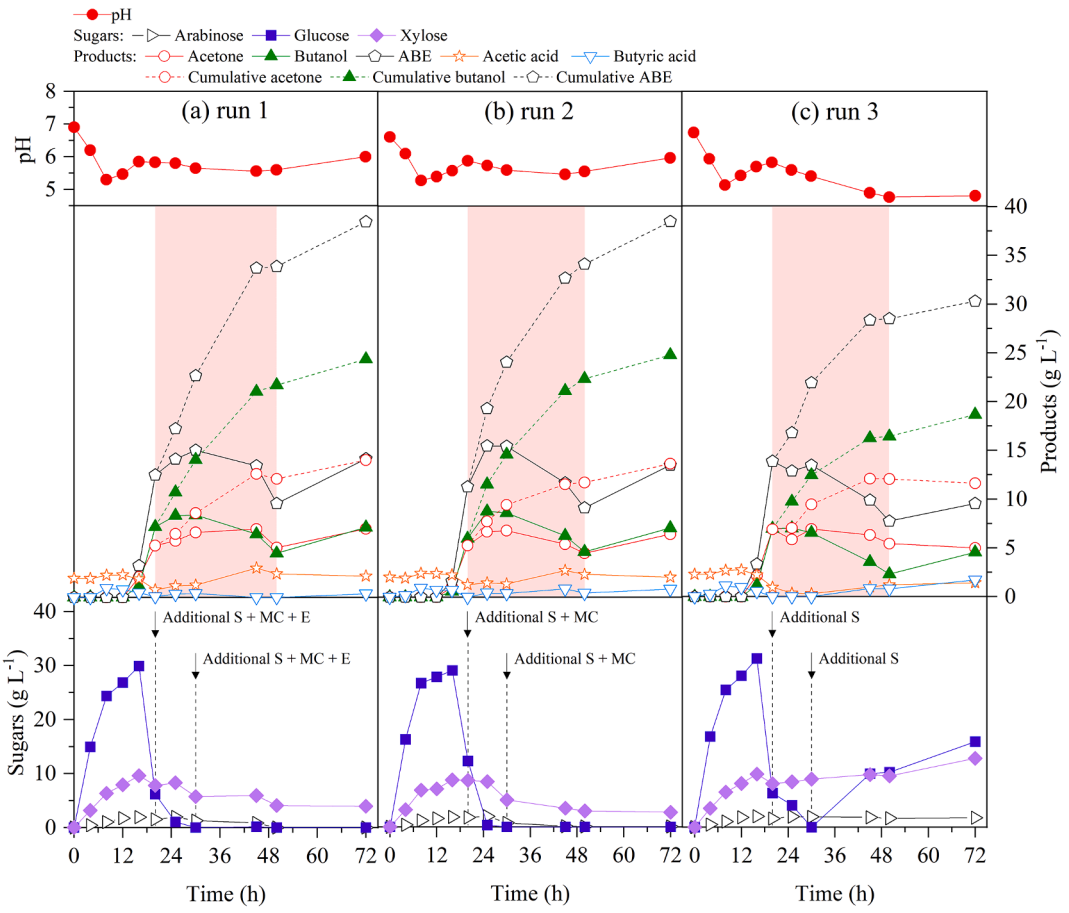

Fig. 4. Time course of the fed-batch SSFR in a 2-L reactor. Additional solid (S) was added with medium compounds (MC) and enzyme (E) in run 1, with MC but without E in run 2 and without MC and E in run 3. Red shaded region indicates the application period of gas striping. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

potential inhibitors, only total phenolic compounds were found at the end of the process, but at a non-inhibitory concentration (0.70 g L^{-1}). The fed-batch strategy allowed processing in 72 h a total RS loading of 18.4% (w/v), which is equivalent to a gradual feeding of 120 g L^{-1} of sugars (89.00 g L^{-1} glucose, 26.72 g L^{-1} xylose and 4.28 g L^{-1} arabinose) according to the saccharification control where 60 g L^{-1} of sugars were obtained when half of the solid loading was used. As fed-batch SSF successfully avoided product inhibition of saccharification, with this approach the low sugar yield typically found in SHF when high sugar concentrations are processed (Zhu et al., 2005) is completely eliminated. While glucose accumulation stopped after 25 h, the xylose concentration remained at 5.59 ± 1.77 g L^{-1} from 25 h to 72 h because a balance was established between hydrolytic enzyme activity and bacterial metabolism. *C. beijerinckii* DSM 6422 consumed 97% of the released sugars (100% of glucose and arabinose and 85% of xylose). After analyzing the condensates from gas stripping, a total volume of 150 mL was recovered at the end of the process with an average butanol concentration of 32.21 ± 3.91 g L^{-1} and ABE of 45.14 ± 4.52 g L^{-1}. The cumulative concentrations of solvents were calculated by dividing the mass of the solvents in the reactor plus those recovered in the condenser by the reactor volume. Cumulative ABE production at 72 h was thus 38.36 g L^{-1} (butanol:acetone mass ratio = 1.73), giving an ABE productivity of 0.533 g $L^{-1}h^{-1}$. Of the ABE solvents, 24.33 g L^{-1} were butanol, resulting in a butanol productivity of 0.338 g $L^{-1}h^{-1}$ lower than the average butanol stripping rate (0.562 g $L^{-1}h^{-1}$) enabling the decrease on the butanol concentration in the reactor. The butanol selectivity, defined elsewhere (Qureshi et al., 1992), was calculated to 4.96 ± 0.97. In comparison with butanol selectivity (between 4 and 22.57) reported by other gas stripping studies (Qureshi et al., 2014, Qureshi et al., 2006), the selectivity of this experiment is in the lower range, so that it could be improved decreasing the gas flow rate or the cooling temperature as it has been suggested by Xue et al. (2014). Compared with the butanol (12.24 g L^{-1}) and ABE (21.09 g L^{-1}) production in the batch SSF (Fig. 3b), the values obtained in run 1 nearly doubled, as did the productivity, as the fermentation time was the same (72 h), improving slightly the butanol-biomass ratio up to 64.0 g-butanol kg-raw RS^{-1}. Considering the results obtained in the system without product recovery, the butanol yield fell by 11% (from 0.237 to 0.210 g g of consumed $sugar^{-1}$) and that of ABE by 19% (from 0.408 to 0.330 g g of consumed $sugar^{-1}$). The moderate reduction in butanol and ABE yield could be associated with the fact that the cells could increase their maintenance energy expenditure when they are continuously exposed to a sub-lethal butanol concentration (Branska et al., 2018). SEM images showed a high amount of long-chain *C. beijerinckii* DSM 6422 cells adhered to the altered surface of the RS at 20 h. The micro-fibrous cellulose structures were used as an immobilization carrier, although no biofilm was found. Unlike other *Clostridium* strains such as *C. acetobutylicum*, *C. beijerinckii* does not form biofilms as it has a weak cell-to-cell communication system (Liu et al., 2018). At 50 h, SEM images showed a small number of cells on the biomass support and some were identified as mother cells for spores, since they showed a swollen clostridial shape. The broth became viscous at this time, probably due to the autolysis promoted by sporulation as a natural survival strategy for the long-term (Branska et al., 2018). A third feeding of RS was added at 72 h but the fermentation did not progress further (data not shown). From SEM images it could be seen that the outer surface of the rice straw has been significantly affected by the

enzymatic degradation after 72 h. Compared with the reported ABE-SSF studies, the final solid loading used (18.4% w/v) was much higher than those typically found in the literature, which range from 7.4 to 13% (Dong et al., 2016; Gallego et al., 2015; Guan et al., 2016; Qi et al., 2019; Razali et al., 2018). Only Li et al. (2016) were able to efficiently use a similar amount of lignocellulosic feedstock (17.5% w/w of steam-exploded corn straw) by means of an intensification method such as periodic peristalsis.

Once it had been shown that fed-batch SSFR could efficiently produce butanol from lignocellulosic waste, two additional experiments were planned with the aim of improving the process's economics (runs 2 and 3). In run 2 (Fig. 4b), the medium compounds ratio over solids was kept as in run 1, but no extra doses of enzyme were incorporated, which halved the final enzyme loading (9.95 FPU g-dw^{-1}). The same cumulative butanol (24.80 g L^{-1}) and ABE (38.47 g L^{-1}, butanol:acetone mass ratio = 1.81) production were attained by using half the enzyme dosing, giving a butanol productivity of 0.344 g L^{-1}h^{-1}, an ABE productivity of 0.534 g L^{-1}h^{-1} and a butanol-biomass ratio of 65.3 g-butanol kg-raw RS^{-1}. No remarkable differences were found in the profiles of pH, products and sugars between run 1 (Fig. 4a) and run 2 (Fig. 4b). These results suggest that the activity of cellulase, β-glucosidase and hemicellulose combined in the Cellic® CTec2 blend lasted for the whole fed-batch SSFR without further enzyme addition, which is a great economic saving. It is important to consider that, depending on the selected substrate, the hydrolytic enzyme can account for over 12% of operational costs in an integrated process such as SSFR (Qureshi et al., 2020). Simultaneously repeated hydrolysis and fermentation (SRHF) is another strategy for enzyme recycling, where sequential cycles of both stages are conducted in parallel in separate vessels (Zhao et al., 2019). Unlike cellulases and xylanases, β-glucosidase without a cellulose-binding module does not bound to lignocellulosic substrates (Várnai et al., 2011), so that β-glucosidase is gradually lost in SRHF, notably reducing the glucose concentration over the saccharification cycles (Zhao et al., 2019). One of the advantages of the process of this work is thus the use of a sole dosage of enzyme blend to hydrolyze sequential biomass additions. The fermentation profile in run 3, in which neither extra medium compounds nor enzymes were added after inoculation, is shown in Fig. 4c. While in previous experiments pH was recovered from 30 h (second biomass addition) to the end of fermentation, in this case pH decreased from 5.41 to 4.81, so that not adding ammonium acetate adversely impacts the buffering capacity, as this compound was used as a solvent precursor. Although under these pH conditions the undissociated acids did not reach inhibitory concentrations, 25% of the sugars released from the RS remained unused, showing that nutrients were limited. The incomplete uptake of sugars by bacterial cells led to a 20% reduction in the cumulative production of butanol (18.67 g L^{-1}), ABE (30.30 g L^{-1}, butanol:acetone mass ratio = 1.60) and butanol-biomass ratio (49.1 g-butanol kg-raw RS^{-1}) at 72 h compared to previous experiments. The results of this work confirm that the nitrogen, vitamins, amino acids and minerals supplied by the initial yeast extract dosage were not enough. In a previous study on SSF optimization from microwave-pretreated RS, the regression model predicted a 17% reduction in butanol production by *C. beijerinckii* DSM 6422 (from 5.28 to 4.37 g L^{-1}) when yeast extract was halved from 4 to 2 g L^{-1} (Valles et al., 2020).

Results show that the fed-batch SSF configuration previously investigated to produce ethanol from biomass (Shengdong et al., 2006; Wang et al., 2013) is also a feasible configuration for butanol production from lignocellulosic waste. By reducing the overall process time by combining saccharification, fermentation and product recovery, high butanol productivities with one enzyme dosage were achieved. Indeed, higher productivities than the alternative configurations for improving butanol productivity such as fed-batch SHF or continuous fermenters were obtained. For example, fed-batch SHF with product recovery has been used to ferment sugarcane-sweet sorghum juices, obtaining a productivity of 0.13 g L^{-1}h^{-1} (Rochón et al., 2017), while suspended-growth cell continuous processes reported productivities ranging from 0.18 to 0.23 g L^{-1}h^{-1} with lignocellulosic waste (Al-Shorgani et al., 2019; Van Hecke and De Wever, 2017). Fed-batch SSFR is in fact a promising configuration for improving ABE productivity, as it avoids the strain degeneration problem of a continuous process and SHF substrate inhibition. However, further research is required to avoid autolysis and extend the operational time by implementing, for instance, a fermenter bleeding strategy.

4. Conclusions

An advanced configuration based on simultaneous saccharification and fermentation combined with gas stripping (SSFR) was shown to be a suitable configuration to produce butanol from lignocellulosic waste. SSFR operated in fed-batch quickly processed high solid loadings while avoiding both substrate and product inhibition, allowing butanol productivity as high as 0.344 g L^{-1}h^{-1} (titer of 24.80 g L^{-1}) by processing an RS loading of 18.4% (w/v) in the short operational time (hydrolysis + fermentation) of 72 h. The enzyme was efficiently used, as no additional enzyme dosing was necessary, thus improving the process's economic viability.

CRediT authorship contribution statement

Alejo Valles: Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Javier Álvarez-Hornos:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Project administration. **Miguel Capilla:** Methodology, Visualization. **Pau San-Valero:** Conceptualization, Methodology. **Carmen Gabaldón:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.126020.

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E-supplementary data

Fed-batch simultaneous saccharification and fermentation including *in-situ* recovery for enhanced butanol production from rice straw

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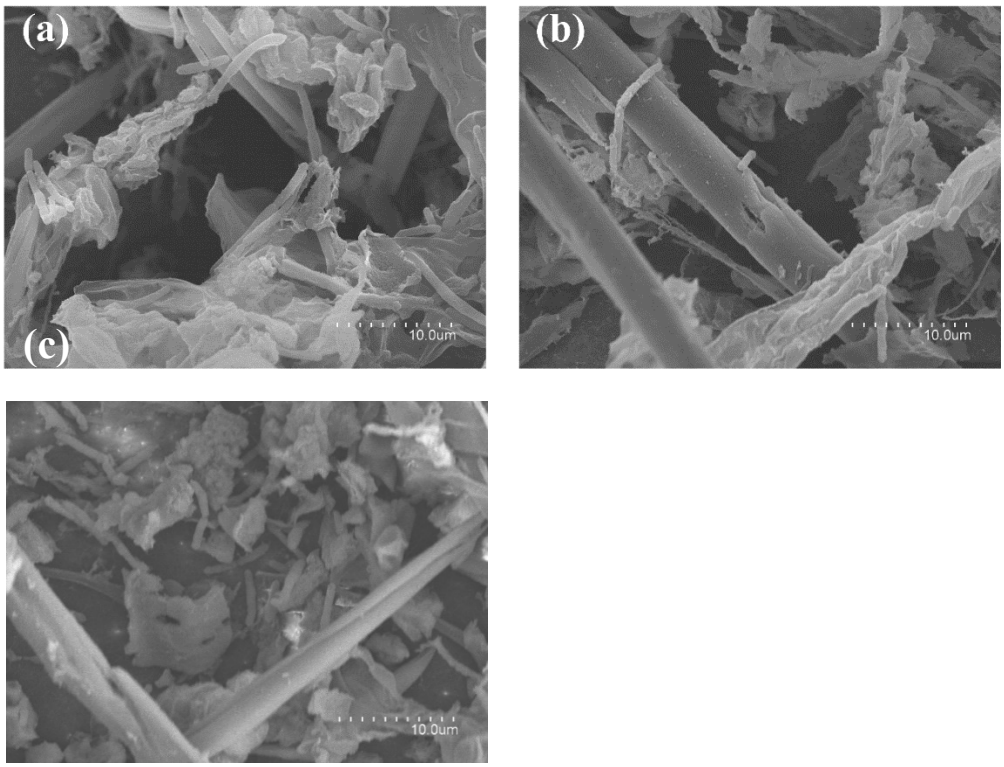

Figure S1. SEM images of cells of *C. beijerinckii* DSM 6422 grown on the surface of alkaline-pretreated RS at 20 h (a), 50 h (b) and 72 h (c) of the fed-batch SSFR. The photographs have been taken at 3000 magnification.

Table S1. CCD experimental matrix along with the values of pH and acids (acetic and butyric, g L^{-1}) at 24 and 72 h.

Run	Real values[a]		24 h			72 h		
			pH	Total Acids (g L^{-1})		pH	Total Acids (g L^{-1})	
	X_1	X_2		Acetic	Butyric		Acetic	Butyric
1	5.0	7.0	6.06	1.34	0.60	5.34	1.42	1.32
2	11.0	7.0	6.19	1.00	1.44	6.00	1.33	0.00
3	5.0	23.0	5.53	1.38	1.12	5.21	1.62	1.28
4	11.0	23.0	6.28	0.93	0.87	5.98	0.96	0.00
5	3.8	15.0	5.59	1.27	0.98	5.29	1.26	1.71
6	12.2	15.0	6.14	0.95	1.89	5.92	1.50	0.00
7	8.0	3.7	5.76	1.71	0.61	5.21	2.33	1.55
8	8.0	26.3	6.12	0.98	1.49	5.81	1.22	0.00
9 – 13	8.0	15.0	5.85 ± 0.23	1.21 ± 0.19	1.08 ± 0.26	5.46 ± 0.13	1.56 ± 0.23	0.64 ± 0.25

[a] X_1: solid loading (% w/v); X_2: enzyme loading (FPU g-dw^{-1}).


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