UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

PROGRAMA DE MAESTRÍA Y DOCTORADO EN INGENIERÍA

AMBIENTAL – RESIDUOS SÓLIDOS

EVALUACIÓN DE LOS IMPACTOS ECONÓMICOS Y AMBIENTALES DE LA PRODUCCIÓN DE BIOCOMBUSTIBLES LÍQUIDOS DE SEGUNDA GENERACIÓN BAJO EL CONCEPTO DE BIORREFINERÍA

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN INGENIERÍA

PRESENTA:

M. EN I. DIANA GONZÁLEZ TENORIO

TUTORA PRINCIPAL

DRA. IDANIA VALDEZ VAZQUEZ, INSTITUTO DE INGENIERÍA

Santiago de Querétaro, Querétaro, 2022

JURADO ASIGNADO:

Presidente:	Dr. Arturo Sanchez Carmona, CINVESTAV
Secretario:	Dra. Leonor Patricia Güereca Hernández, Instituto de Ingeniería
1 er. Vocal:	Dr. Carlos Eduardo Molina Guerrero, Universidad de Guanajuato.
2 do. Vocal:	Dr. Germán Buitrón Méndez, Instituto de Ingeniería
3 er. Vocal:	Dra. Idania Valdez Vazquez, Instituto de Ingeniería

Lugar donde se realizó la tesis: Laboratorio de Investigación de Procesos Avanzados de Tratamiento de Aguas, Unidad Académica Juriquilla-Querétaro, Instituto de Ingeniería, UNAM.

TUTORA DE TESIS:

Dra. Idania Valdez Vazquez,

FIRMA

Agradecimientos académicos

A CONACYT, por la beca 421114 otorgada para el desarrollo de este trabajo.

Al financiamiento recibido por DGAPA-UNAM proyectos PAPIIT (No. IA102018, IN102721) y al Fondo de Sustentabilidad Energética 2014-05 (CONACYT SENER) Centro Mexicano de Innovación en Bionergía, Clúster de Bioalcoholes (No. 249564).

A DAAD, Agencia Alemana por la beca otorgada para la asistencia a las escuelas de verano (2018) e invierno (2020).

Agradecimientos personales

A Dios, por todo lo que me ha dado.

A mi familia, que siempre está conmigo.

A mí comité tutoral, que me asesoró para el desarrollo de este trabajo.

A la M. Gloria Moreno-Rodríguez, al M. Jaime Pérez Trevilla y al I. Ángel Avizua Hernández Huerta por el apoyo técnico especializado para el desarrollo de esta tesis.

Al Dr. Martín Barragán y a la Dra. Marisol Pérez, por sus enseñanzas y buenos consejos.

A la Dra. Sandra Quiñonez y al equipo médico por cuidar de mi salud, siempre con una sonrisa.

A Areli, Marisol, Mariana, Edith y Stefany.

A mis compañeros lignocelulósicos y de Lipata por las sonrisas y momentos agradables.

Índice

Resumen

Esta investigación se enfocó en la producción de biobutanol a partir de residuos de maíz mediante una serie de bioprocesos consolidados en cascada, todos ellos empleando consorcios microbianos mixtos como biocatalizadores. La investigación se dividió en dos partes, en la primera se estudiaron los bioprocesos consolidados mediante ensayos de laboratorio y en la segunda, los estudios se enfocaron en la integración de los bioprocesos mediante esquemas de biorrefinamiento para evaluar los posibles impactos en diferentes dominios: económico, ambiental y social. Los bioprocesos estudiados fueron: (1) proceso de fermentación acidogénica para solubilizar la fracción de hemicelulosa; (2) proceso de fermentación acetona, butanol y etanol (ABE) a partir de la celulosa residual; y (3) fermentación de residuos y gases H_2/CO_2.

En el proceso de fermentación acidogénica, la microbiota nativa del rastrojo de maíz solubilizó la hemicelulosa produciendo hidrógeno, dióxido de carbono (CO_2) y ácidos grasos volátiles (AGV). Este bioproceso fue operado durante 195 días en modo semicontinuo con una productividad máxima de hidrógeno de 87 mL/L·día, y producción de AGV de 8.5 g/L de acetato, 3.7 g/L de butirato y 2.2 g/L de propianato. La microbiota nativa estuvo dominada por bacterias de los géneros *Enterococcous, Prevotella, y Megasphaera*. Después, los sólidos gastados del proceso de acidogénesis se usaron como sustrato en el proceso de fermentación ABE utilizando un consorcio microbiano.

En la fermentación ABE se utilizaron diferentes diseños experimentales para evaluar el efecto del pH (6.5 y 7.5), composición de los AGV (acetato, propianato y butirato), y las estrategias de bioaumentación y evolución adaptativa en laboratorio (EAL) sobre la concentración de butanol. Se encontró que a un pH de 7.5, y solo en presencia de ácido butírico, el consorcio microbiano produjo butanol una concentración máxima de 120 mg/L. Cuando se empleó la bioaumentación del consorcio microbiano con *Clostridium saccharobutylicum* BAA-117, y en presencia de ácido butírico, la concentración máxima de butanol aumentó a 610 mg/L. Los mejores resultados se lograron al aplicar la estrategia EAL, donde el consorcio microbiano fue incubado en diferentes concentraciones crecientes de butanol con rastrojo de maíz enriquecido

en celulosa como sustrato. El consorcio microbiano que se adaptó a concentraciones de butanol de 3.25 g/L logró producir 13.8 g/L de butanol. El análisis de la comunidad microbiana reveló que este consorcio se enriqueció con *Bacteroides*, *Caproiciproducens*, *Anaerofilium*, *Acetanaerobacterium*, una especie no identificada del Orden Clostridia UCG-014, y *Pseudomonas*. Las funciones atribuidas a este consorcio son la hidrólisis de los polisacáridos (celulosa), producción de AGV que después fueron asimilados para producir butanol, así como desarrollar mecanismos para la tolerancia a solventes.

El tercer bioprocesos estudiado, la fermentación de los gases producidos en la acidogénesis (H_2 y CO_2), tuvo el objetivo de evitar que sean emitidos a la atmósfera, y utilizarlos como materia prima para obtener un mayor rendimiento de butanol, o bien, producir otros productos de fermentación con mayor valor en el mercado, como el caproato. En la fermentación de gases, se empleó un gas modelo de H_2 y CO_2 a una relación de 75:25 v/v, y se evaluó el efecto del medio de cultivo (medio A con una fuente de carbono/nitrógeno orgánicas, y el medio B compuesto de vitaminas, minerales y trazas de metales) y el origen del inóculo (lixiviados y digestato provenientes de un proceso de acidogénesis, y lodos anaerobios). Los resultados mostraron que al emplear los lodos anaerobios y el medio B se obtuvo la mayor concentración de butanol de 889 mg/L; al contrario, al emplear los lixiviados junto con el medio B se logró obtener hasta 6 000 mg/L de caproato.

Después de la experimentación, la siguiente parte de la investigación consistió en la simulación de los bioprocesos en tres modelos de biorrefinería. Los tres modelos de biorrefinería propuestos fueron: 1) el modelo base (MB) conformado por la fermentación acidogénica, la fermentación ABE, la digestión anaerobia, cogeneración y purificación de productos con butanol, calor y electricidad como principales productos; 2) el modelo con biofijación de CO_2 conformado por los mismos bioprocesos del modelo base con la adición de la fermentación de gases provenientes de la acidogénesis para la producción adicional de butanol (modelo con 2 escenarios, MCO_2ButOH-I y MCO_2ButOH-II); y 3) el modelo con biofijación de CO_2 con la misma configuración que el modelo 2, con la diferencia de que la fermentación de gases está dedicada a la producción de caproato (modelo con 1 escenario, MCO_2Cap). Todos los modelos de biorrefinería tuvieron una alimentación de 1000 toneladas/año de residuos de maíz para fines de comparación. Se realizó un análisis técnico-económico para determinar la viabilidad

del proyecto y se determinó el costo total de producción empleando una asignación económica. Después, se realizó un análisis multidimensional (económico, ambiental y social) para determinar el modelo de biorrefinería más sustentable. En el análisis económico se consideraron los indicadores de costo total de producción (CTP) y rendimiento; en el análisis ambiental se consideraron los indicadores de consumo de agua, la calidad de agua del efluente, las emisiones de CO_2, y la eficiencia energética; y en el análisis social se consideró el indicador de número de empleados. Estos indicadores fueron monetizados empleando la unidad funcional de USD por tonelada de sustrato para la comparación entre los escenarios.

El CTP en MB fue de 1.23 USD/$kg_{butanol}$. En MCO_2ButOH-I y MCO_2ButOH-II, la conversión de CO_2 en butanol adicional no tuvo un impacto importante en el CTP. No obstante, en MCO_2Cap el CTP se redujo a 0.18 USD/$kg_{butanol}$, gracias a la asignación económica. El mejor desempeño ambiental lo tuvo MCO_2ButOH-II debido a que el CO_2 fue convertido en butanol adicional lo que mejoró notablemente la eficiencia energética. El indicador social fue mejorado en los modelos de biofijación de CO_2 debido a los procesos y flujo adicionales que requieren un mayor número de personas empleadas. En lo global, el acoplamiento de la biofijación de CO_2 en MB para la producción adicional de butanol resultó en el modelo más sustentable debido principalmente a su mejor desempeño ambiental.

El estudio profundo de cada uno de los tres bioprocesos dieron origen a tres artículos científicos. Los resultados del proceso de fermentación acidogénica, de la bioaumentación para incrementar la concentración de butanol y de la producción del butanol por el proceso de fermentación de gases están reflejados en el resumen del capítulo 3.0 y en el artículo publicado: Butanol production coupled with acidogenesis and CO_2 conversion for improved carbon utilization. Biomass Conv. Bioref. https://doi.org/10.1007/s13399-020-00805-y.

Los resultados de la EAL se muestran en el capítulo 4.0 y por medio del artículo en revisión para ser publicado Adaptive laboratory evolution applied to an acidogenic microbial community for enhanced butanol production from corn stover cellulose. Para la fermentación de gases los resultados de la producción de ácidos grasos de cadena media se muestran en el capítulo 5.0 y en artículo publicado Fermentation of organic wastes and CO_2 + H_2 off-gas by microbiotas

provides short-chain fatty acids and ethanol for n-caproate production. J CO_2 utilization 42,101314. https://doi.org/10.1016/j.jcou.2020.101314.

Por último, los resultados de la simulación de los bioprocesos se muestran en el resumen del capitulo 6.0 y del artículo sometido Sustainability analysis of single- and multi-product lignocellulosic biorefineries using biological CO_2 fixation.

Capítulo 1.0 Introducción general

1.1 Marco teórico

Los rendimientos anuales de biomasa lignocelulósica se estiman en más de 220 billones de toneladas, lo que equivale de 60 a 80 billones de toneladas de petróleo crudo [1]. Entre las ventajas del uso de la lignocelulosa están su alta disponibilidad, sus bajos costos y sus reducidos impactos negativos al ambiente. Entre los biocombustibles que se pueden obtener de la biomasa, se encuentran el etanol, el hidrógeno y el butanol. El butanol posee características fisicoquímicas superiores en comparación con el etanol, además, puede mezclarse con gasolina y utilizarse directamente como carburante en automóviles [2]. Una gran desventaja que presentan el uso de este biocombustible es en su producción como el alto costo de la biomasa, el bajo rendimiento en la producción y los altos costos de producción en comparación con los costos de producción del butanol proveniente del petróleo [3]. A continuación, mediante la siguiente revisión bibliográfica se explican más a fondo estas cuestiones.

1.1.1 Butanol

El butanol (o alcohol butílico o 1-butanol) es un alcohol que tiene aplicaciones como solvente de pinturas, cosméticos, formulación de detergentes, medicamentos, antibióticos, hormonas y vitaminas. También es empleado como un bloque de construcción químico para la producción de moléculas más complejas como acrilato, metacrilato de butilo, disolventes de pintura, polímeros, lacas, plásticos y esmaltes [2, 4].

Este alcohol es muy prometedor como combustible alternativo al poseer características superiores a la gasolina comparado contra otros alcoholes como el etanol o el metanol (Tabla 1.1) [5]. Además de que este alcohol se puede usar directamente como combustible en motores de combustión interna en forma de mezcla o en su forma pura en los motores sin modificación en los automóviles [4] y su combustión genera bajas emisiones de gases de efecto invernadero [6]. El butanol se produce por procesos químicos como síntesis de oxo, síntesis de Reppe y crotonaldehído y por vía fermentativa.

Tabla 1.1 Comparación de las propiedades físicas y químicas de combustibles líquidos

Propiedades	Butanol	Etanol	Metanol	Gasolina
Fórmula molecular	C_4H_9OH	C_2H_5OH	CH_3OH	C_4-C_{12}
Peso molecular	74	46	32	95-120
Punto de ebullición (°C)	118	78	65	200
Punto de enfriamiento (°C)	-89	-114	-97	-40
Densidad (kg / m^3)	810	790	796	760
Calor de Vaporización (MJ /kg)	0.43	0.92	1.20	0.36
Densidad energética (MJ / L)	30	19	16	32
Relación aire y combustible	12	9	7	15
No. Cetano	25	5-8	3.8	5-20
Clasificación número de octano	96	107	106	95
Numero de octanaje de motor	78	89	92	90
Punto de inflamabilidad (°C)	35	13	12	42
Temperatura de autoignición (°C)	397	423	463	257
Contenido de oxígeno (%)	22	46	50	95-120

1.1.2 Biomasa lignocelulósica

Los biocombustibles se clasifican por el sustrato empleado para su producción. Se les denomina de primera generación (1G) cuando son cultivos dedicados a la alimentación como el maíz y la caña de azúcar; de segunda generación (2G) cuando son residuos orgánicos forestales y agrícolas (este último llamado biomasa lignocelulósica); los de tercera generación (3G) son algas y la cuarta generación (4G) plantas y microorganismos modificados genéticamente [7]. La biomasa lignocelulósica está formada en su interior por celulosa, hemicelulosa y lignina. La celulosa (30-50%), es una sustancia cristalina con alto grado de polimerización y esta revestida por la hemicelulosa (o xilano) (15-35%) y por la lignina (10-20%). Por la conformación de la biomasa ligoncelulósica, la celulosa tiene una difícil accesibilidad (Figura 1), por lo que se requieren tratamientos previos para su disponibilidad. La composición de la lignocelulosa es propia de cada materia prima y está contenida en las diferentes partes de la biomasa como en los tallos, granos, raíces, frutos y pajas [8].

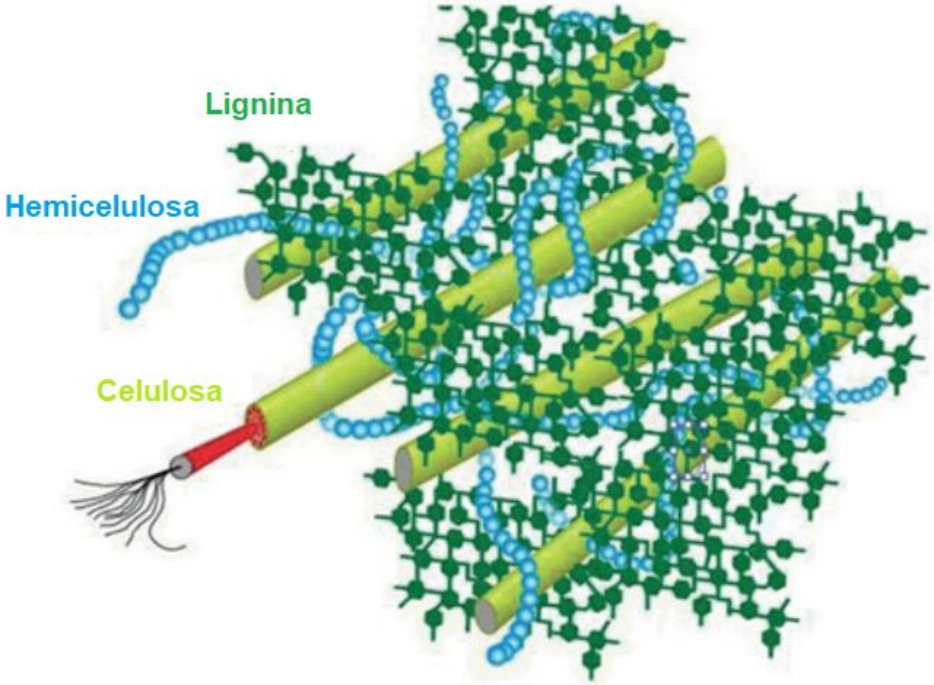

Figura 1. Componentes de la lignocelulosa [9].

1.1.3 Biorrefinerías de segunda generación para la producción de butanol

Según la Agencia Internacional de Energía una biorrefinería se define como el conjunto de métodos para el procesamiento sostenible de biomasa en una variedad de productos de base biológica comercializables (ingredientes de alimentos, productos químicos y materiales) y energía (combustibles, energía y calor) en un contexto de economía circular y valorizando múltiples productos [10].

Respecto a la producción de butanol en la literatura se proponen biorrefinerías multiproductos [11, 12] y se destaca que constan de las etapas de pretratamiento, fermentación ABE y la separación y purificación de los solventes ABE. Otras innovaciones son la conjunción de estos procesos a través de bioprocesos consolidados [13], fermentación ABE con purificación integrada [14] o adicción de nuevos procesos como la biofijación de CO_2 a través de la fermentación de gases [15].

1.1.3.1 El *pretratamiento* tiene como objetivos la reducción de tamaño físico de la lignocelulosa, la destrucción de las estructuras recalcitrantes de la lignocelulosa (lignina y hemicelulosa), la

accesibilidad de los microorganismos a la celulosa, el incremento del área superficial, la porosidad y la cristalinidad. El pretratamiento es el paso crítico y entre los más costosos de la producción de los biocombustibles (20-60% del costo total) [16]. Los pretratamientos pueden ser físicos (trituración mecánica), fisicoquímicos (explosión de vapor, explosión de fibra de amoniaco), químicos (ozonólisis, hidrólisis ácida, hidrólisis alcalina, métodos biológicos (hidrólisis enzimática) [17].

1.1.3.2 La *fermentación* que consta de dos etapas secuenciales, la primera fase es la acidogénesis donde ocurre el crecimiento exponencial de los microorganismos a un pH entre 6.5 a 7.0 y la conversión de glucosa (hexosa) en acetato, butirato, dióxido de carbono (CO_2) e hidrógeno (H_2). La segunda etapa es la solventogénesis, en esta etapa el pH desciende a entre 4.5 y 5.0, y los AGV se convierten en los solventes acetona, butanol y etanol (ABE) y en los gases H_2 y CO_2 [18].

El butanol es producido naturalmente por la bacteria del genero Clostridium spp, vía fermentación ABE con un radio de 3:6:1; entre las especies de *Clostridium* que se reportan para la producción de butanol están: *Clostridium acetobutylicum, Clostridium beijerinkii, Clostridium saccharobutylicum* y *Clostridium saccharoperbutylacetonicum*.

La producción de butanol puede ser por cepas silvestres o por cepas modificadas genéticamente. Si se emplea glucosa como sustrato y cepas silvestres, las concentraciones de butanol oscilan de 0.02 g/L (con *Clostridium perfrigens*) hasta de 15.21 g/L (con *Clostridium beijerinckii*) [4]. Así mismo, cuando se emplean las especies modificadas genéticamente como *Clostridium acetobutylicum* JB200 produce butanol a una concentración de 20.30 g/L, por lo que se pueden obtener valores más altos con clostridia modificadas, sin embargo, no pueden se puede mantener la tasa de crecimiento celular, ni una fermentación continua [19].

Las bajas concentraciones de butanol se deben a la baja capacidad de las bacterias para tolerar altas concentraciones de butanol, en consecuencia, las bacterias se intoxican y el metabolismo celular cesa al alterar los componentes fosfolípidos de la membrana celular provocando una fluidez de la membrana y la alteración de las funciones como el proceso del transporte y absorción de la glucosa [20, 21].

Entre las innovaciones para aumentar las concentraciones de butanol está emplear el Bioproceso Consolidado (BPC) en el que ocurren de forma simultánea cuatro procesos: producción de enzimas sacarolíticas (celulasa y hemicelulasa), la hidrólisis enzimática de carbohidratos y la fermentación de los azúcares de 6 carbonos (glucosa, manosa y galactosa) y de los azúcares de 5 carbonos (xilosa y arabinosa) [22]. Las características del BPC al llevarse a cabo todos los procesos en un solo reactor, son los menores de inversión y de operación; menor el consumo de agua potable y menor cantidad de agua residual, así como mayores rendimientos en la producción de biocombustibles [22].

En el BPC los procesos son llevados a cabo por consorcios microbianos integrados por microorganismos con actividades metabólicas complementarias, es decir, hay una sinergia entre los microorganismos, interacción y no competición [23], junto con una gran diversidad celular y la posibilidad de diferentes vías de transformación [24].

1.1.3.4 Separación y purificación de los solventes ABE. Posterior a la fermentación ABE, la siguiente etapa es obtener los solventes acetona, butanol y etanol; entre los métodos de separación están la destilación, filtración por membrana, adsorción, membrana, extracción líquido-líquido y pervaporación. La selección del método depende del costo, requerimiento de energía, estabilidad, selectividad, facilidad de operación e integración al proceso de fermentación [3, 14]. Los costos de esta etapa son del 10 al 50 % del costo de inversión y operación de las biorrefinerías [3, 13].

1.1.3.5 Biofijación de CO_2 (Fermentación de gases), la fermentación biológica de gases en una tecnología prometedora al producir solventes como el etanol, el butanol y el hexanol, productos químicos de alto valor agregado y contribuir a la captura de gases de efecto invernadero mediante bacterias acetogénicas a través de vías metabólicas como la vía Wood-Ljungdahl [25, 26]. Estas bacterias tienen la capacidad para fijar el CO_2 (electrón aceptor) utilizando H_2 (electrón donante) para producir los productos [27]. Los gases que se emplean en este proceso son el monóxido de carbono, el dióxido de carbono, el hidrógeno y el metano. Este proceso está influenciado por los acetógenos, tipo de reactor, composición del gas, componentes del medio, parámetros de funcionamiento como la temperatura y la presión [26].

1.1.4 Análisis de sustentabilidad

Las biorrefinerías son un medio para agregar valor a través de una producción más sustentables, por lo que la sustentabilidad de los productos de base biológica se examina exhaustivamente y se evalúan empleando diferentes esquemas a través de sus impactos de producción [28].

Una biorrefinería se debe diseñar y operar de manera sustentable, en otras palabras, económicamente viable, socialmente responsable y ambientalmente favorable [29]. Entre las evaluaciones se consideran los criterios de sustentabilidad durante la etapa del diseño de las biorrefinerías resultando en diseños alternativos y obteniéndose mejora en los desempeños generales [28]; en los dominios económicos y ambientales representa una un gran reto para la ingeniería de procesos [30].

Diversas publicaciones se enfocan en evaluar la sustentabilidad en por diferentes análisis como empleando la cuantificación de indicadores (o métricas) [29], como el evaluado por [31] et al. (2016) que cuantifican las emisiones de gases de efecto invernadero y otro ejemplo un trabajo enfocado a los costos del butanol en el que se evalúan diferentes materias primas y en una configuración de la biorrefinería que no considera pretratamiento, hidrólisis ni enzimas celulíticas y con recuperación de las solventes in situ, los costos de venta mínimo resultantes fueron entre $ 1.28 a $ 1.40 USD por litro de butanol [32].

También se tienen publicaciones de análisis bidimensionales que abarcan dos criterios de la sustentabilidad, en específico en el ámbito económico y ambiental como son [33], (2017) que analizaron los impactos ambientales de la producción de butanol por vía bioquímica incorporando los costos de los productos y otro ejemplo, son análisis técnico-económicos [13, 32, 34]. El trabajo reportado por [13] et al, (2018) que hacen un análisis técnico-económico de una biorrefinería avanzada de residuos lignocelulósicos para la producción de butanol. Sin embargo, no se tienen análisis de sustentabilidad para biorrefinerías de butanol de 2G, solo se tienen de 1G enfocadas en el etanol [35].

1.1.5 Referencias

[1] Ren N., Wang A., Cao G., Xu J., Gao L., (2009) Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges, Biotechnology Advances, 27 (6): 1051-1060, https://doi.org/10.1016/j.biotechadv.2009.05.007.

[2] Belletante S., Monstastruc L., Negny S. D. & Domenech S. (2016) Optimal design of an efficient, profitable and sustainable biorefinery producing acetone, butanol and ethanol: Influence of the *in-situ* separation on the purification structure Biochemical Engineering Journal. 116 (15): 195–209. https://doi.org/10.1016/j.bej.2016.05.004.

[3] Veza I., Said M. F. M., Latiff Z. A., (2021) Recent advances in butanol production by acetone-butanol-ethanol (ABE) fermentation, Biomass and Bioenergy, 144: 105919, https://doi.org/10.1016/j.biombioe.2020.105919.

[4] Patil R. C., Suryawanshi P. G., Kataki R., Goud V. V., (2019) Chapter 8 - Current challenges and advances in butanol production, Editor(s): Rai M., Ingle A. P., Sustainable Bioenergy, Elsevier: 225-256, https://doi.org/10.1016/B978-0-12-817654-2.00008-3.

[5] Huzir N. M., Maniruzzaman M., Aziz A., Ismail S.B., Abdullah B., Mahmood N. A. N., Umor N.A., Muhammad S. A. F. S., (2018) Agro-industrial waste to biobutanol production: Eco-friendly biofuels for next generation, Renewable and Sustainable Energy Reviews, 94: 476-485, https://doi.org/10.1016/j.rser.2018.06.036.

[6] Liu X., Xue S., Ikram R., Zhu C., Shi Y., He M., (2021), Improving the viscosity and density of n-butanol as alternative to gasoline by blending with dimethyl carbonate, Fuel, 286 (2): 119360, https://doi.org/10.1016/j.fuel.2020.119360.

[7] Kumar B., Bhardwaj N., Agrawal K., Chaturvedi V., Verma P., (2020) Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept, Fuel Processing Technology, 199: 106244, https://doi.org/10.1016/j.fuproc.2019.106244.Liu et al., 2015.

[8] Dharmaraja J., Shobana S., Arvindnarayan S., Vadivel M., Atabani A.E., Pugazhendhi A., Kumar G., (2020), Chapter 5 - Biobutanol from lignocellulosic biomass: bioprocess strategies, Editor(s): Yousuf A., Pirozzi D., Sannino F., Lignocellulosic Biomass to Liquid Biofuels, Academic Press, 169-193, https://doi.org/10.1016/B978-0-12-815936-1.00005-8.

[9] Wang S., & Zhongyang L. (2016) Chapher 1. Biomass components and characteristics" Book Pyrolysis of Biomass, Berlin, Boston: De Gruyter, 2016, pp. 1-32. https://doi.org/10.1515/9783110369632-003

[10] Dragone G., Kerssemakers A. A.J., Driessen J. L.S.P., Yamakawa C. K., Brumano L.P., Mussatto S. I., 2020, Innovation and strategic orientations for the development of advanced biorefineries, Bioresource Technology, 302: 122847, https://doi.org/10.1016/j.biortech.2020.122847.

[11] Xia F., Liu H., Lu J., Lv Y., Zhai S., An Q., Cheng Y., Wang H., (2019) An integrated biorefinery process to produce butanol and pulp from corn straw, Industrial Crops and Products,140: 111648, https://doi.org/10.1016/j.indcrop.2019.111648.

[12] Pratto B, Chandgude V, Ruy de Sousa, Gonçalves C. A. J., Bankar S., (2020) Biobutanol production from sugarcane straw: Defining optimal biomass loading for improved ABE fermentation, Industrial Crops and Products, 148: 112265, https://doi.org/10.1016/j.indcrop.2020.112265.

[13] Valdez-Vazquez, I. and Sanchez, A. (2018), Proposal for biorefineries based on mixed cultures for lignocellulosic biofuel production: a techno-economic analysis. Biofuels, Bioprod. Bioref., 12: 56-67. https://doi.org/10.1002/bbb.1828.

[14] Patraşcu I., Bîldea C. S., Kiss A. A., (2017) Eco-efficient butanol separation in the ABE fermentation process, Separation and Purification Technology, 177: 49-61, https://doi.org/10.1016/j.seppur.2016.12.008.

[15] González-Tenorio D., Muñoz-Páez K.M., Valdez-Vazquez I., (2020), Butanol production coupled with acidogenesis and CO2 conversion for improved carbon utilization, Biomass Conv. Bioref. https://doi.org/10.1007/s13399-020-00805-y.

[16] Morone A. & Pandey R.A. (2014) Lignocellulosic biobutanol production: Gridlocks and potential remedies, Renewable and Sustainable Energy Reviews 37: 21–35. https://doi.org/10.1016/j.rser.2014.05.009.

[17] Liu Y, Lyu Y., Tian J., Zhao J., Ning Ye, Zhang Y., Chen L., (2021a) Review of waste biorefinery development towards a circular economy: From the perspective of a life cycle assessment, Renewable and Sustainable Energy Reviews, 139: 110716, https://doi.org/10.1016/j.rser.2021.110716.

[18] Nanda S., Golemi-Kotrab D., McDermottb J. C., Dalai A. K. Gökalp I., Kozinski J. A., (2017). Fermentative production of butanol: Perspectives on synthetic biology, New Biotechnology 37 (B): 210–221. https://doi.org/10.1016/j.nbt.2017.02.006.

[19] Liu, S., Qureshi, N., & Hughes, S. R. (2017). Progress and perspectives on improving butanol tolerance. *World journal of microbiology & biotechnology*, *33*(3), 51. https://doi.org/10.1007/s11274-017-2220-y.

[20] Xue C., Cheng C., (2019) Chapter Two - Butanol production by Clostridium, Editor(s): Li Y., Ge X., Advances in Bioenergy, Elsevier, 4: 35-77, https://doi.org/10.1016/bs.aibe.2018.12.001.

[21] Lee S. Y., Park J. H., Jang S. H., Nielsen L. K., Kim J., & Jung K. S. (2008). Fermentative butanol production by Clostridia. Biotechnology and bioengineering, 101(2), 209–228. https://doi.org/10.1002/bit.22003.

[22] Lynd L. R., van Xyl H. W., McBride J. E., Laser M. (2005) Consolidated bioprocessing of cellulosic biomass: an update, Biochemical engineering. 16 (5):577–583. https://doi.org/10.1016/j.copbio.2005.08.009.

[23] Wu C.-H., Tran D.-T., Shih M.-C., Li W.-H., Wu C.-F., (2011), Mixed culture fermentation from lignocellulosic materials using thermophilic lignocellulose-degrading anaerobes, Process Biochemistry, 46, (2): 489-493, https://doi.org/10.1016/j.procbio.2010.09.024.

[24] Zhang H. & Wang X., (2016) Modular co-culture engineering, a new approach for metabolic engineering, Metabolic Engineering, 37: 114-121, https://doi.org/10.1016/j.ymben.2016.05.007.

[25] Phillips J. R., Atiyeh H. K., Tanner R. S., Torres J. R., Jyotisna Saxena, Mark R. Wilkins, Raymond L. Huhnke, (2015), Butanol and hexanol production in Clostridium carboxidivorans syngas fermentation: Medium development and culture techniques, Bioresource Technology, 190: 114-121. https://doi.org/10.1016/j.biortech.2015.04.043.

[26] Sun X., Atiyeh H. K., Huhnke R. L., Tanner R. S. (2019) Syngas fermentation process development for production of biofuels and chemicals: A review, Bioresource Technology Reports, 7: 100279, https://doi.org/10.1016/j.biteb.2019.100279.

[27] Ragsdale S. W. & Pierce E., (2008) Acetogenesis and the Wood–Ljungdahl pathway of CO_2 fixation, Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1784 (12): 1873-1898, https://doi.org/10.1016/j.bbapap.2008.08.012.

[28] Palmeros P. M., Osseweijer P., Posada D. J. A., (2017), Sustainable biorefineries, an analysis of practices for incorporating sustainability in biorefinery design, Industrial Crops and Products,106: 105-123, https://doi.org/10.1016/j.indcrop.2016.08.052.

[29] Sacramento-Rivero, J.C. (2012), A methodology for evaluating the sustainability of biorefineries: framework and indicators. Biofuels, Bioprod. Bioref., 6: 32-44. https://doi.org/10.1002/bbb.335.

[30] Martinez-Hernandez E., Campbell G., Sadhukhan J., (2013) Economic value and environmental impact (EVEI) analysis of biorefinery systems, Chemical Engineering Research and Design, 91(8): 1418-1426, https://doi.org/10.1016/j.cherd.2013.02.025.

[31] Väisänen S., Havukainen J., Uusitalo V., Havukainen M., Soukka R., Luoranen M., (2016) Carbon footprint of biobutanol by ABE fermentation from corn and sugarcane, Renewable Energy, 89: 401-410, https://doi.org/10.1016/j.renene.2015.12.016.

[32] Qureshi, N., Lin, X., Liu, S., Saha, B. C., Mariano, A. P., Polaina, J., Ezeji, T. C., et al. (2020). Global View of Biofuel Butanol and Economics of Its Production by Fermentation from Sweet Sorghum Bagasse, Food Waste, and Yellow Top Presscake: Application of Novel Technologies. *Fermentation*, *6*(2), 58. MDPI AG. Retrieved from http://dx.doi.org/10.3390/fermentation6020058.

[33] Brito M. & Martins F. (2017) Life cycle assessment of butanol production, Fuel, 208:476-482, https://doi.org/10.1016/j.fuel.2017.07.050.

[34] Sanchez A., Valdez-Vazquez I., Soto A., Sánchez S., Tavarez D., (2017), Lignocellulosic n-butanol co-production in an advanced biorefinery using mixed cultures, Biomass and Bioenergy, 102: 1-12, https://doi.org/10.1016/j.biombioe.2017.03.023.

[35] López-Ortega M. G., Guadalajara Y., Junqueira T. L., Sampaio I. L.M., Bonomi A., Sánchez A., (2021) Sustainability analysis of bioethanol production in Mexico by a retrofitted sugarcane industry based on the Brazilian expertise, Energy, 232: 121056, https://doi.org/10.1016/j.energy.2021.121056.

1.2 Hipótesis

Una biorrefinería lignocelulósica multiproducto que aprovecha los subproductos líquidos y gaseosos como el CO_2 para producir energía (butanol, vapor y electricidad) y productos de alto valor agregado será más sustentable que una biorrefinería monoproducto dedicada a producir energía.

1.3 Objetivos

1.3.1 Objetivo general

Evaluar los impactos económicos, ambientales y sociales de la producción de biobutanol a partir de residuos de maíz en una biorrefinería basada en bioprocesos consolidados empleando consorcios microbianos.

1.3.2 Objetivos particulares

1. Producir H_2 y ácidos grasos volátiles (acético y butírico) en una fermentación acidogénica a partir de rastrojos de maíz y utilizando consorcios microbianos. (https://doi.org/10.1007/s13399-020-00805-y).

2. Determinar experimentalmente el valor de pH y la concentración de ácidos grasos volátiles que incremente la concentración de butanol en la fermentación ABE a partir del rastrojo de maíz en un bioproceso consolidado (https://doi.org/10.1007/s13399-020-00805-y).

3. Aumentar la concentración del butanol en una fermentación ABE a partir de la fracción de celulosa de rastrojos de maíz mediante dos estrategias: la bioaumentación y la evolución adaptativa en laboratorio de microbiotas nativas (para bioaumentación: (https://doi.org/10.1007/s13399-020-00805-y).

4. Determinar el efecto del medio de cultivo y del tipo de inóculo sobre la producción de ácidos grasos de cadena media y solventes a partir de la fermentación de gases H_2/ CO_2 provenientes de un proceso de acidogenésis (https://doi.org/10.1016/j.jcou.2020.101314).

5. Establecer un modelo de biorrefinería en cascada que sea sustentable para la producción de butanol y otros productos químicos a partir de rastrojos de maíz.

Capítulo 2.0 Estrategia general de trabajo

En la Figura 2.1 se muestra un diagrama con la estrategia general de trabajo empleada para el desarrollo de esta investigación que constó de dos fases subsecuentes e interdisciplinarias, que se describen brevemente a continuación.

2.1 Parte experimental (Fase uno)

Con el propósito de cumplir con los cuatro primeros objetivos de esta investigación mediante diferentes experimentos en laboratorio, se estudiaron los procesos de fermentación acidogénica, fermentación ABE y fermentación de gases para la producción de butanol a partir de residuos de maíz.

2.1.1 Actividad 1, Fermentación acidogénica *(Objetivo 1)*

El proceso de fermentación acidogénica convierte la fracción de hemicelulosa en AGV, en específico acetato y butirato, una fase gaseosa compuesta de H_2/CO_2, quedando un digestato enriquecido en celulosa y lignina.

Este bioproceso utilizó un consorcio microbiano nativo como biocatalizador, por lo que el sustrato se alimenta sin una esterilización previa. Los experimentos se realizaron con un volumen efectivo de 600 mL, en condiciones mesofílicas, a una carga orgánica de 9 g_{ST}/L-día, con un tiempo de retención hidráulico (TRH) de 6.6 días, en modo semicontinuo durante 195 días. Se realizó el seguimiento de la producción de hidrógeno, producción de AGV, y la evolución de la estructura microbiana por secuenciación masiva.

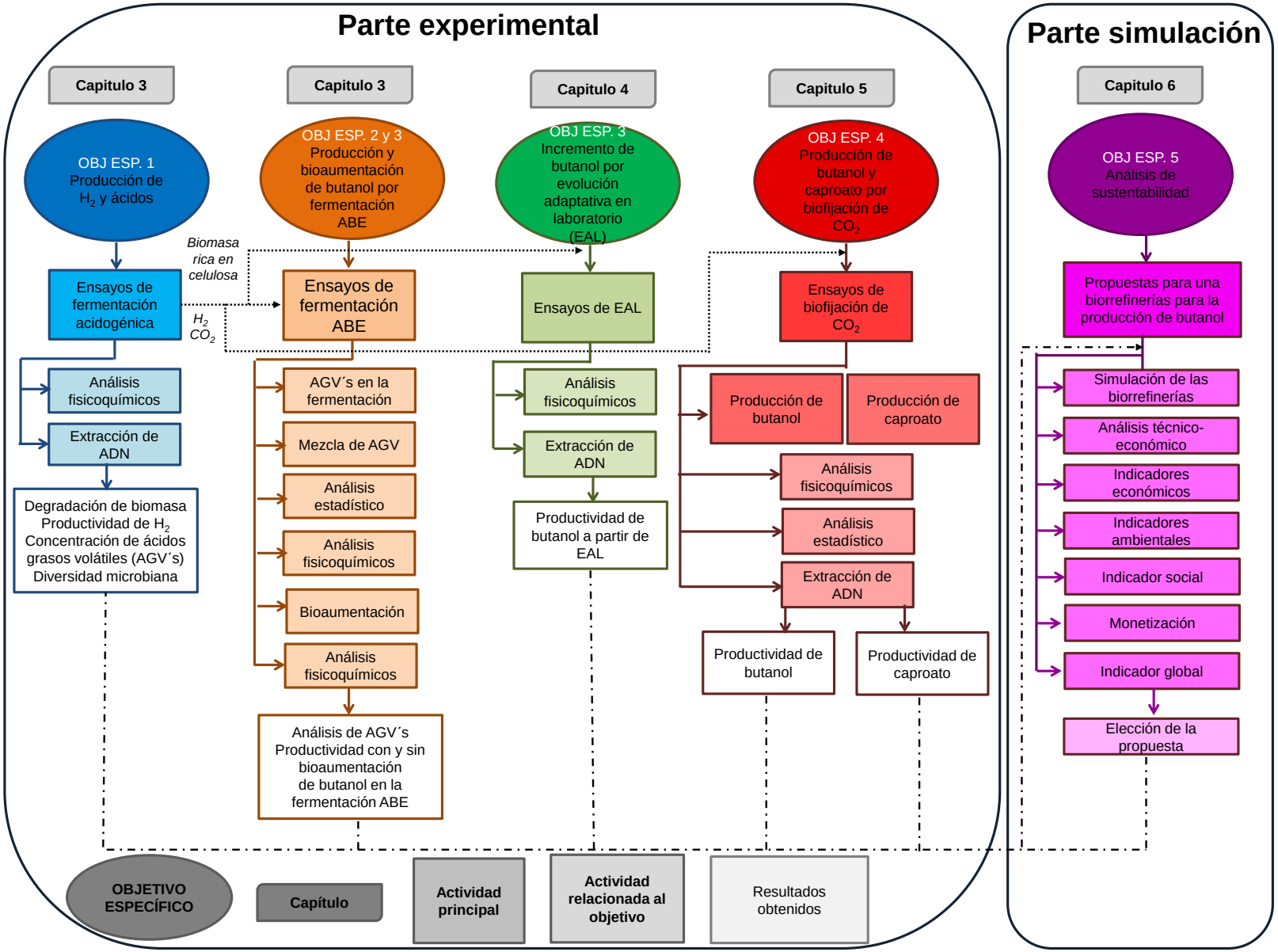

Figura 2.1 Estrategia de trabajo empleada en esta investigación.

Los digestatos obtenidos de esta actividad fueron lavados y almacenados para utilizarse como sustrato en las siguientes actividades.

2.1.2 Actividad 2, Fermentación ABE *(Objetivo 2)*

El proceso de fermentación ABE produce los solventes acetona-butanol-etanol a partir de los digestatos enriquecidos en celulosa provenientes de la fermentación acidogénica. En este bioproceso se estudió dos variables que influyen en la fermentación ABE y producen mayor concentración de butanol: el pH y la relación de los AGV.

En la fermentación ABE se utilizó un consorcio microbiano tratado térmicamente como biocatalizador. Los experimentos se realizaron con un volumen efectivo de 100 mL, en condiciones mesofílicas, a una carga sólidos totales de 10%, con un tiempo de incubación de 7 días (modo lote). Se utilizó un diseño experimental de mezclas centroide simplex para estudiar el efecto de la relación de AGV (ácido acético, propiónico y butírico) sobre la producción de butanol, a pH 6.5 y 7.5. Se analizó la concentración de los solventes al final de la incubación.

2.1.3 Actividad 3, Estrategias para incrementar la concentración de butanol en la fermentación ABE *(Objetivo 3)*

Como resultado de la Actividad 2, se encontró que el consorcio microbiano fue capaz de producir butanol en una concentración muy baja de 230 mg/L. Por tanto, la Actividad 3 estuvo enfocada en aplicar dos estrategias para incrementar la concentración de butanol por el consorcio microbiano.

2.1.3.1 Actividad 3.1, Estrategia de bioaumentación

La fermentación ABE se realizó nuevamente utilizando como sustrato los digestatos de la acidogénesis, y como inóculo un consorcio microbiano bioaumentado con *Clostridium saccharobutylicum* BAA-117. Los experimentos se realizaron con un volumen efectivo de 100 mL, en condiciones mesofílicas, a una carga sólidos totales de 10%, con un tiempo de incubación de 7 días (modo lote). Además, se adicionaron mezclas de AGV, se acuerdo a los

mejores resultados encontrados en la Actividad 2. La bioaumentación se realizó un día después de la incubación para permitir el establecimiento de condiciones anaerobias. Se analizó la concentración de butanol al final de la incubación.

2.1.3.2 Actividad 3.2, Estrategia de evolución adaptativa en laboratorio

La fermentación ABE se realizó empleando residuos de maíz tratados enriquecidos en celulosa, y como inóculo consorcios microbianos adaptados a cuatro concentraciones de butanol que incrementaron durante doce periodos de incubación. Los experimentos se realizaron con un volumen efectivo de 100 mL, en condiciones mesofílicas, a una carga sólidos totales de 10%, con un tiempo de incubación de 7 días (modo lote). Se analizó la concentración de AGV y butanol al final de cada incubación, y la evolución de la estructura microbiana por secuenciación masiva.

2.1.4 Actividad 4, Fermentación de gases H_2/CO_2 *(Objetivo 4)*

El proceso de fermentación de gases produce solventes y ácidos grasos de cadena media, este bioproceso emplea como sustrato H_2 y CO_2, gases generados en la acidogénesis. En este bioproceso, se estudiaron dos variables que influyen en la fermentación de gases: el tipo de inóculo y el medio de cultivo.

La fermentación de gases se realizó empelando una mezcla sintética de H_2/CO_2. Los experimentos se realizaron con volumen gaseoso útil de 200 mL, condiciones misofilias, agitación orbital de 150 rpm, en modo semicontinuo durante 233 días. Se analizó el consumo de H_2, CO_2 y concentración de productos de fermentación (ácidos grasos volátiles y ácidos grasos de cadena media), y la evolución de la estructura microbiana por secuenciación masiva.

2.2 Parte de simulación (Fase dos)

Con el propósito de cumplir con el último objetivo de esta investigación, se trabajó en la simulación de diferentes propuestas de biorrefinerías virtuales para la producción de butanol a partir de residuos lignocelulósicos.

2.2.1 Actividad 5.1, Diseño conceptual de una biorrefinería modelo *(Objetivo 5)*

Inicialmente, se tomó como modelo base (MB) la biorrefinería propuesta por Valdez Vazquez & Sanchez, (2017) para producción de butanol. Este MB fue alimentado los resultados experimentales obtenidos en las Actividades 1-4 en las etapas de acidogénesis y fermentación ABE.

2.2.2 Actividad 5.2, Propuesta de biorrefinerías modificadas para la fijación de CO_2

Posteriormente, el MB fue modificado para el aprovechamiento del CO_2 presente en los gases de fermentación, y utilizarlo como fuente de carbono para obtener un mayor rendimiento en la biorrefinería, en específico, para producir butanol adicional y caproato (reportado con un mayor valor económico que el butanol). Se hicieron dos nuevas propuestas de biorrefinerías que acoplan la biofijación de CO_2 al MB.

2.2.3 Actividad 5.3, Análisis de sustentabilidad

Primero, se realizó el análisis técnico-económico para los tres modelos de biorrefinerías propuestos en las Actividades 5.1 y 5.2 empleando el software SuperPro Designer® v 11.0 (Intelligen, Inc., NJ, USA) utilizando parámetros del contexto mexicano en 2021. Este software permite calcular el VPN, los costos totales de producción, costos de operación, costos de inversión y con ello determinar dos indicadores económicos: rendimiento y costo total de producción (Sanchez et al., 2014).

Después, con los balances de masa obtenidos se cuantificaron cuatro indicadores ambientales: gases de efecto invernadero, consumo de agua, calidad del efluente y eficiencia

energética. Así mismo, se obtuvo como indicador social la generación de empleos. A continuación, los indicadores de los dominios económico, ambiental y social se normalizan para su comparación con el MB, como esquema de referencia. Finalmente, se hizo una monetización para comparar entre los tres modelos de biorrefinería propuestos y se cuantificó un indicador global para determinar el modelo de biorrefinería más sustentable.

En los siguientes capítulos se muestran los resultados y discusiones de estos. Inicialmente en cada capítulo tiene un resumen que entrelaza los objetivos y resultados con los otros capítulos y después se muestra el artículo publicado o enviado para su publicación.

Capítulo 3.0 Resultados. Fermentación acidogénica acoplada a la fermentación ABE y a la fermentación de gases

El capítulo 3 estuvo dedicado al cumplimiento del objetivo específico 1, es decir, producir H_2, acetato y butirato durante la fermentación acidogénica, así como el objetivo específico 2, determinar el efecto del pH y relación de AGV sobre la producción de butanol durante la fermentación ABE. Adicionalmente, se presentan los resultados del objetivo específico 3, donde mediante una estrategia de bioaumentación de incrementó la concentración de butanol en la fermentación ABE utilizando consorcios microbianos como biocatalizador.

La primera motivación del trabajo fue conocer el efecto del cambio de cultivar de maíz sobre el desempeño a largo plazo de un reactor de fermentación acidógenica operado en modo semi-continuo para la producción de hidrógeno, CO_2 y AGV. El reactor de fermentación acidogénica fue operado durante 195 días en un reactor con volumen útil de 600 mL, y utilizando como inóculo el consorcio nativo de maíz. Durante este tiempo de operación, el reactor se alimentó con dos cultivares, el cultivar Dk-2038 en los días 1 al 94 y el cultivar VS-536 en los días 95–195. En términos generales, se observó que la productividad de H_2 incrementó gradualmente hasta lograr valores máximos de 87 ± 3 mL/L-d. El cambio de cultivar al día 95 disminuyó por un corto periodo el porcentaje de H_2, pero sin modificar la productividad de hidrógeno. La producción de acetato siempre fue mayor que la producción de butirato y propionato. A partir del día 120 de operación, la producción de AGV incrementó a concentraciones de 8.5 g/L de acetato, 3.7 g/L butirato y 2.2 g/L de propionato. El consorcio nativo del maíz experimentó un cambio importante durante el establecimiento de la fermentación acidogénica. Originalmente, el consorcio nativo estuvo formado principalmente por *Agrobacterium*, *Klebsiella*, y *Pantoea*. Durante las primeras etapas de la fermentación acidogénica, el consorcio nativo estuvo dominado por *Propionibacterium*, *Clostridium*, y *Lactobacillus*. No obstante, a partir del cambio del cultivar en la alimentación, tres especies ganaron dominancia, *Enterococcus*, *Prevotella*, y *Megasphaera*. De este modo se concluye que el cambio de cultivar en la alimentación del reactor no tuvo un efecto sobre la productividad de hidrógeno, pero sí tuvo un efecto sobre la estructura de la comunidad microbiana.

La experiencia ganada con la operación del reactor de fermentación acidogénica permitió conocer el perfil de AGV producidos, y obtener sólidos gastados enriquecidos en celulosa, ambos a utilizarse como sustratos en un segundo bioproceso, la fermentación ABE. Por estudios anteriores se conoce que el butirato tiene un efecto positivo sobre la producción de butanol, al ser un intermediario directo. Sin embargo, la fermentación acidogénica produce una mezcla de AGV donde el acetato es el principal metabolito y no el butirato. Por tanto, para acoplar los procesos de acidogenesis y fermentación ABE fue necesario conocer el efecto de mezclas de AGV sobre la producción de butanol. Por ello, se utilizó un diseño de mezclas para conocer el efecto de mezclas de acetato, butirato y propionato sobre la producción de butanol, utilizando los digestatos de la acidogénesis como fuente de carbono. Los ensayos fueron realizados en reactores en lote con un volumen útil de 100 mL y un consorcio microbiano mixto como inóculo.

Los resultados fueron contundentes, el consorcio microbiano produjo butanol sólo en presencia de butirato, mientras que en una mezcla de AGV enriquecida con acetato, la producción de butanol fue cercana a cero. Esto plantea la necesidad de modificar la composición de AGV en la fermentación acidogénica, con el fin de evitar la inhibición de la producción de butanol "*aguas abajo*" en la biorrefinería. De otro modo, sería necesario lavar los sólidos gastados que se usan como sustrato en la fermentación ABE lo que evitar la inhibición incrementará el consumo de agua de forma notable. En paralelo, se encontró que el consorcio produjo una cantidad no satisfactoria de butanol, solo 120 mg/L. Por tanto, se aplicó la estrategia de bioaumentación con *Clostridium saccharobutylicum* BAA-117, una especie que produce butanol a concentraciones mayores a 10 g/L. A pesar de que la concentración de butanol se incrementó a 610 mg/L, aún sigue siendo una concentración muy por debajo de la deseada para explotación industrial (al menos 15 g/L).

Finalmente, en este capítulo se reportan los resultados de la fermentación de H_2 y CO_2, gases producidos en la fermentación acidogénica. La motivación de estudiar este bioproceso es para acoplar la fermentación de gases con la acidogénesis y evitar la emisión de gases de efecto invernadero, y en paralelo incrementar el rendimiento de productos al convertir el CO_2 en butanol. Se estudió el efecto del tipo de inóculo y medio de cultivo sobre la producción de butanol a partir de una mezcla sintética de H_2/CO_2. Los experimentos se hicieron con un

volumen útil en el espacio gaseoso de 200 mL con una mezcla sintética de H_2/CO_2 de 75:25 v/v. Se encontró que la mayor producción de butanol de 889 mg/L se logró con un medio de cultivo con minerales traza y un inóculo enriquecido con Spirochaetales, *Pseudomonas*, *Enterobacter*, y *Proteiniphilum*.

El estudio de los bioprocesos de acidogénesis, fermentación ABE y fermentación de gases permitió determinar la factibilidad técnica de integrarlos en un esquema de biorrefinería para lograr un máximo aprovechamiento de los residuos lignocelulósicos. Sin embargo, se identificaron algunas barreras que deben estudiarse posteriormente.

Primero, para el acoplamiento de la acidogénesis con la fermentación ABE es importante modificar la composición de AGV para que el butirato sea el principal AGV, y con ello, se favorezca la producción de butanol "*aguas abajo*" en la biorrefinería. Segundo, en la fermentación ABE la estrategia de bioaumentación fue ineficiente en incrementar la producción de butanol a los niveles deseados (> 10 g/L). Por tanto, se deben estudiar otros factores que afectan la bioaumentación, o bien, explorar otras estrategias para incrementar la capacidad del consorcio para producir butanol. Finalmente, la fermentación de la mezcla H_2/CO_2 fue exitosa en producir butanol por un consorcio autotrófico. Sin embargo, se debe estudiar el efecto de otras composiciones de H_2/CO_2 que asemejen a las corrientes gaseosas generadas en la acidogénesis donde el H_2 no sobrepasa el 50% en la mezcla.

Referencia del trabajo publicado

González-Tenorio, D., Muñoz-Páez, K.M. & Valdez-Vázquez, I., (2020) Butanol production coupled with acidogenesis and CO_2 conversion for improved carbon utilization. Biomass Conv. Bioref. https://doi.org/10.1007/s13399-020-00805-y.

3.1 Abstract

Butanol production from lignocellulosic biomass has a low overall yield due to carbon loss in the form of xylose during the biomass pretreatment and CO_2 during the fermentation processes. This research presents a cascading process for producing butanol coupling three bioprocesses. First, an indigenous microbial community performed the direct acidogenesis of raw corn stover producing an H_2-CO_2 gas stream and volatile fatty acids (VFA) using the most biodegradable fraction. The acidogenesis process had maximum hydrogen productivity of 87 mL/L·d and VFA production of 8.5 g/L of acetic acid, 3.7 g/L of butyric acid, and 2.2 g/L of propionic acid. The acidogenesis process experienced a species succession with early colonizing bacteria dominated by *Lactobacillus* being replaced with more succeeding microbial groups dominated by *Enterococcous, Prevotella,* and *Megasphaera*.

Second, the spent solids of corn stover were used for producing acetone-butanol-ethanol (ABE) using a mixed culture bioaugmented with *Clostridium saccharobutylicum*. A simplex centroid mixture design served to elucidate the effects of adding different mixtures of acetic, butyric, and propionic acids on butanol production. Pure butyric acid improved three times the butanol titer compared to the control treatment with no acid addition (610 mg/L versus 230 mg/L of butanol, respectively). Opposite, acetic and propionic acids inhibited the butanol production. Finally, additional butanol was produced using an H_2-CO_2 gas stream, where the type of inoculum and culture medium affected the process. An inoculum enriched with Spirochaetales, *Pseudomonas, Enterobacter,* and *Proteiniphilum* grown on a culture medium with trace metals reached the highest butanol titer of 889 mg/L. This cascading process improved the carbon utilization by producing butanol from VFA and CO_2, and not only from cellulose.

Keywords: Acetogens; Bioaugmentation; Butanol; Cascading process; *Clostridium saccharobutylicum*; CO_2 conversion.

3.2 Introduction

Lignocellulosic biomass, which is by far one of the most abundant wastes generated on the planet, has a complex composition and can, therefore, be transformed into various types of biofuels and intermediate chemicals for the generation of high-value products [1]. Butanol fuel has gained interest as substitute for conventional fuels and as an industrial solvent for the manufacture of antibiotics, vitamins, and hormones. Biochemically, clostridial species perform acetone-butanol-ethanol (ABE) fermentation, typically using only the cellulose fraction of lignocelluosic feedstock, with an overall butanol yield of approximately 100 g/kg of butanol [2]. The low yield of ABE fermentation is due to carbon loss during the processing of the lignocellulosic substrate where the xylan fraction is discarded during the pretreatment, and CO_2 produced by bacteria during fermentation is emitted into the atmosphere in the form of off-gases.

A previous study coupled the acidogenesis of alkaline pretreated rice straw producing volatile fatty acids (VFAs) with 72% butyric acid with ABE fermentation from enzymatic hydrolysates [3]. With this two-stage fermentation process, the butanol yield was increased to 230 g/kg; however, the authors still used chemicals (NaOH) and costly enzymes for the processing of the lignocellulosic substrate. As a new perspective, the fermentation process (also referred to as acidogenesis) serves as the first stage that converts raw lignocellulosic feedstock fermenting extractives and xylan – the most biodegradable fraction – to H_2-CO_2 and VFAs, leaving unfermented spent solids, including cellulose [4]. Direct acidogenesis performs as a consolidated bioprocess where the production of hydrolytic enzymes and fermentation occurs together, thus direct acidogenesis serves as a soft pretreatment and circumvents the need for costly enzymes. Nevertheless, past research on the direct acidogenesis of waste streams is focused mainly on the utilization of food wastes, activated sludge, and microalgal biomass [5], with limited knowledge of the performance of direct acidogenesis of lignocellulosic feedstocks in continuous reactors. The experience gained has been mainly with batch reactors, where the acidogenesis of bagasses and wheat straw produced VFAs enriched with acetic acid, > 70% [4, 6]. Therefore, the coupling of these two bioprocesses—direct acidogenesis with ABE fermentation requires knowledge about the effects of different compositions of VFAs on butanol production.

Biorefineries are facilities focused on the valorization of lignocellulosic biomass; although they generate biofuels and bioproducts, they also emit a large amount of carbon in the form of CO_2

from fermentation and cogeneration processes [7]. An innovative way to valorize these off gas streams is to use them as substrates to produce alcohols through gas fermentation. In this context, acetogens ferment CO_2 plus H_2 to VFAs and alcohols via the Wood Ljungdahl pathway [8]. To date, previous studies have made efforts to determine the best conditions to produce alcohols in batch gas fermentation and mainly utilized artificial syngas streams and pure cultures of *Clostridium carboxidivorans*. The principal parameters tested have included the type of medium and cultivation temperature obtaining butanol titers over 1.6 g/L [9–12]. The medium selected should provide enough nutrients for the process, and the costs of the medium for high scale production should be considered. In this regard, Phillips et al. [9] studied a limited defined medium, and with a selected cultivation protocol, 1.0 g/L butanol was produced from a model syngas. Shen et al. [10] optimized the concentration of trace metals and cultivation temperature using an artificial steel mill off-gas as a substrate, and a concentration of 1.67 g/L butanol was observed. Ramió-Pujol et al. [11] also observed the highest butanol concentration of 1.1 g/L at 25 °C using a mixed culture. More recently, Chakraborty et al. [12] used an acclimatized mixed culture, and a maximum concentration of 0.6 g/L butanol was detected in syngas fermentation. Despite the interest in the use of waste gas streams for alcohol production, to the best of our knowledge, no one has studied butanol production from the off-gases of acidogenesis and ABE processes containing CO_2-H_2 (unlike syngas, which contains CO), an approach that is receiving increased interest to reduce green gas emissions in biorefineries

3.3 Methodology

3.3.1 First stage: direct acidogenesis

3.3.1.1 Substrate and inoculum

The raw lignocellulosic substrate consisted of corn stover (*Zea mays* L.). During the operation, two cultivars were used: cultivar Dk-2038 collected in May 2017 from Sinaloa, Mexico (latitude 25°33´55´´ North, longitude -108°28´04´´ West and elevation 50 m, clay soil), and cultivar VS-536 collected in February 2018 from Queretaro, Mexico (latitude 20°45´17´´ North, longitude -100°27´12´´ West and elevation 1950 m, clay soil). The chemical composition was determined using an automated fiber analyzer (ANKOM 200 Fiber Analyzer, ANKOM Technologies, Macedon, NY), according to methods previously reported [13, 14], ie., for cultivar Dk-2038, volatile solids (VS) 0.89 g/g of total solids (TS), extractives 0.23 g/g ST, cellulose 0.41 g/g TS, hemicellulose 0.30 g/g TS, and lignin 0.06 g/g TS; for cultivar VS-536, 0.93 g VS/g TS, extractives 0.22 g/g TS, cellulose 0.44 g/g TS, hemicellulose 0.29 g/g TS, and lignin 0.05 g/g

TS. Corn stover particles were milled and screened to pass a 10-mesh screen. Corn stover particles were stored at environment conditions (28 °C and 27% RH) until its use as the substrate in the acidogenesis reactor. Unsterile corn stover served as the source of inoculum since previous studies have reported that indigenous species have hydrolytic and fermentative activities [11].

3.3.1.2 Procedure and experimental design

The acidogenesis reactor consisted of 1 L anaerobic culture bottles with a working volume of 0.6 L. The reactor was operated in semi-continuous mode with an organic loading rate of 9 gTS/L day, solid retention time of 6.6 days, initial pH of 6.5, and 37 °C. The content of total solids was adjusted with a nutrient solution with the following composition: 4.5 mg urea/g_{TS}, 20 mM potassium phosphate buffer, and 12 mM sodium bicarbonate. The reactor was closed with air in the headspace and fed twice a week in a draw-and-fill mode. The acidogenesis reactor was operated in duplicate.

3.3.2 Second stage: ABE fermentation from spent solids and mixtures of VFA Spent solids generated during the acidogenesis process were used as the carbon source for the ABE fermentation.

These spent solids were supplemented with different synthetic mixtures of VFA to elucidate its effects on the ABE fermentation.

3.3.2.1 Substrate and inoculum

Spent solids (also referred to as digestate) from the acidogenesis reactors were washed with tap water to eliminate the VFA accumulated and then sun-dried (0.99 gVS/gTS). Fresh digestates also served as the source of inoculum, with this aim, a sample of unwashed digestate received a heat shock treatment at 105 °C for 12 h to select spore-forming Clostridia [17].

3.3.2.2 Procedure and experimental design

ABE fermentation reactors consisted of 250 mL glass serum bottles with a working volume of 175 mL. The bottles were loaded with 5.8 g of substrate, 11.6 g of inoculum, and 157.5 mL of a nutrient solution with the following composition (per liter): 2 g urea, 0.5 g KH_2PO_4, and 0.01 g $MgSO_4 \cdot 7H_2O$. The nutrient solution was supplemented with synthetic mixtures of VFA (X_1, acetic acid; X_2, propionic acid; and X_3, butyric acid) to determine its effects on the ABE production using a simplex centroid mixture design (Table 3.1). The experimental design was evaluated at pH 6.5 and 7.5 with a total concentration of acids of 10 g/L. Pure acids correspond to treatments A, B, and C; binary mixtures correspond to treatments D, E, and F; the ternary

mixture corresponds to the treatment G, and treatments H, I, and J correspond to mixtures with a higher ratio of each acid. The control treatment consisted of a bottle with no supplementation of any VFA. All treatments were performed in duplicate. The bottles were capped hermetically with butyl rubber stoppers and sealed with metal rings with air in the headspace. The bottles were incubated at 37 °C for 7 days. The response variables were production of acetone (y_1), ethanol (y_2), and butanol (y_3). The fit of the models to the experimental data was determined by the analysis of variance (ANOVA) and determination coefficient (R^2) by the Design Expert® software v10 (Stat-Ease., Inc., Minneapolis, MN).

3.3.2.3 Bioaugmentation with *Clostridium*

Once the simplex centroid mixture design was performed, some selected treatments were repeated and bioaugmented with Clostridium to improve the butanol titer. Strain and inoculum preparation *Clostridium saccharobutylicum* Keis et al. (ATCC BAA-117) served to improve the ABE fermentation through bioaugmentation. This strain was anaerobically grown on a modified PYG medium under static conditions at 37 °C. The pure culture of *C. saccharobutylicum* was prepared in 150 mL serum bottles with 20 g/L of reducing sugars, pH 7.0, and incubated for 4 days reaching a sugar consumption of 80%.

Table 3.1. Matrix of the simple centroid mixture design for the production of acetone, butanol and ethanol from different mixtures de acetic, propionic and butyric acids.

Run	Concentration (g/L)			pH = 6.5			pH = 7.5		
	Acetic acid (X_1)	Propionic acid (X_2)	Butyric acid (X_3)	Acetone (g/L)	Ethanol (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butanol (g/L)
Control	0	0	0	0.12 ± 0.06	0.32 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.64 ± 0.19	0.00 ± 0.00
A	10	0	0	0.14 ± 0.03	0.40 ± 0.08	0.03 ± 0.00	0.00 ± 0.00	0.42 ± 0.09	0.00 ± 0.00
B	0	10	0	0.22 ± 0.07	0.48 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.09	0.00 ± 0.00
C	0	0	10	0.12 ± 0.00	0.44 ± 0.08	0.08 ± 0.01	0.11 ± 0.01	0.27 ± 0.05	0.12 ± 0.02
D	0	5	5	0.14 ± 0.02	0.35 ± 0.07	0.04 ± 0.02	0.00 ± 0.00	0.40 ± 0.05	0.06 ± 0.01
E	5	0	5	0.17 ± 0.00	0.25 ± 0.15	0.06 ± 0.01	0.00 ± 0.00	0.39 ± 0.09	0.06 ± 0.02
F	5	5	0	0.13 ± 0.01	0.35 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.53 ± 0.1	0.00 ± 0.00
G	3.3	3.3	3.3	0.15 ± 0.02	0.36 ± 0.07	0.03 ± 0.01	0.00 ± 0.00	0.51 ± 0.28	0.01 ± 0.01
H	6.7	1.7	1.7	0.14 ± 0.02	0.51 ± 0.00	0.03 ± 0.01	0.00 ± 0.00	0.22 ± 0.04	0.00 ± 0.00
I	1.7	6.7	1.7	0.21 ± 0.11	0.46 ± 0.11	0.02 ± 0.01	0.00 ± 0.00	0.34 ± 0.00	0.03 ± 0.02
J	1.7	1.7	6.7	0.30 ± 0.01	0.32 ± 0.10	0.05 ± 0.00	0.00 ± 0.00	0.27 ± 0.00	0.05 ± 0.00

3.3.3 Third stage: butanol production from H_2/CO_2

3.3.3.1 Substrate and inoculum

The substrate consisted of a model off-gas (H_2/CO_2 [75:25]). Three inocula were acclimated to ferment H_2/CO_2 to butanol, i.e., (1) an anaerobic sludge from a UASB reactor treating brewery effluents which received a heat shock treatment at 105 °C for 60 min to select spore-forming Clostridia [18], and (2) digestates and (3) supernatants from the acidogenesis process.

3.3.3.2 Procedure and experimental design

Gas fermentation reactors consisted of 250 mL serum bottles with a working volume of 100 mL. The bottles were inoculated with 5 g of each inoculum. A general factorial design of two factors, type of inoculum at three levels and type of culture medium at two levels served to determine its effects on the butanol production. Each condition was tested in duplicate. The culture medium “A” was used during the first 110 days of operation and whose composition per liter was: 4 g urea, 2 g KH_2PO_4, and pH 6.7. After a period of starvation, 50% of culture medium was replaced with the culture medium “B” whose composition was previously reported by Rachbauer et al. [19], pH 7.1. At this point, the feeding was resumed with a hydraulic retention time of 60 days. The bottles were incubated at 37 °C with orbital shaking (150 rpm). The substrate (a mixture of H_2 and CO_2) was added to each reactor once the inoculum had consumed 90% of H_2.

3.3.4 Analytical methods

The volumen of biogas was determined using the brine displacement method. Biogas composition (hydrogen, methane, and carbon dioxide) was determined using a gas chromatograph (SRI 8610C) equipped with a thermal conductivity detector and two packed columns (6′ × 1/8″ silica gel packed column and 6′ × 1/8″ molecular sieve × 13 packed column). The injector and detector temperatures were fixed at 90 °C and 150 °C, respectively. The initial column temperature was 40 °C, which was held for 4 min and then gradually increased to 110 °C at a rate of 20 °C/min. The final column temperature was held for 3 min. The carrier gas was nitrogen at a flow rate of 20 mL/min Muñoz-Paéz et al. [18]. Hydrogen was reported at STP. Centrifugated 0.45-µm-filtered supernatants were analyzed to determine the concentrations of short-chain fatty acids, ethanol, and acetone using a gas chromatograph (Varian 3300) equipped with a flame ionization detector and a 15-m long (0.53 mm id) Zebron ZB-FFAP column. The injector and detector temperatures were fixed at 190 and 210 °C, respectively. The initial column temperature was 45 °C for 1.5 min, which was then increased to 135 °C at a

rate of 8 °C/min. The carrier gas was nitrogen at a flow rate of 9.5 mL/ min. The concentration of butanol was analyzed using an HPLC AGILENT 1260 (Agilent Technologies, CA, USA) equipped with a diode array and refractive index detectors, and an AMINEX HPX-87 H column. A total of 5 mM H_2SO_4 served as eluent at a flow rate of 0.6 mL/min with a column temperature of 50 °C.

3.3.5 Molecular analysis

Selected samples were subjected to DNA extraction using the PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was quantified and sequenced using the Illumina MiSeq Platform, and then, the sequences obtained were analyzed according to Muñoz-Paéz et al. [18].

3.3.6 Statistical analysis

Data were analyzed using a one-way ANOVA and Tukey's test for comparison of means at the 95% confidence interval.

3.4 Results and discussion

3.4.1 Direct acidogenesis of raw corn stover

The acidogenesis reactor was operated for 195 days and fed two corn cultivars, Dk-2038 (on days 1–94) and VS-536 (on days 95–195). During the first 30 days, the hydrogen percentage increased gradually from 11 to 27% on day 67, with average productivity of 42 ± 3 mL/L day (Fig. 3.1a). During the substrate change by day 95, the hydrogen percentage decreased for a short period, but the hydrogen productivity remained almost unchanged. As the acidogenesis process continued, the hydrogen productivities improved until reaching its highest values of 87 ± 3 mL/L day by days 130–150 of operation. The VFA composition was similar among cultivars, and acetic acid was the main fermentation end product, followed by butyric and propionic acids (Fig. 3.1b). Similar to hydrogen productivity, the indigenous microbiota produced more VFAs at the end of the period of operation. The results of the acidogenesis reactor revealed that the performance improved after 100 days of operation. Since the chemical composition among cultivars was quite similar, these differences could be attributed to the activity of the indigenous microbiota. A consistent process of ecological succession characterizes the spontaneous fermentation of food such as fruit, dairy, and plants where early colonizing communities are replaced with more succeeding microbial groups [20]. Before acidogenesis, the indigenous microbiotas among cultivars were similar, 60% of shared genera (Fig. 3.1c, left columns). Some

of these bacteria, such as *Pantoea, Agrobacterium, and Klebsiella*, are common in the phyllosphere of rice, soybean, and wheat [16, 21]. After the first stage of acidogenesis, only 20% of these indigenous bacteria remained, *Lactobacillus, Psychrobacillus*, and *Propionibacterium* were the primary colonizers (Fig. 3c, on day 21). As acidogenesis continued, a second colonizing group dominated by *Clostridium, Lactobacillus, Enterococcus, Acetobacter*, and *Prevotella* replaced the pioneer species (Fig. 3.1c, on days 91–111).

Some of these bacteria could be responsible for the solubilizing process of corn stover; *Enterococcus* and *Prevotella* have xylanase activity thriving on insoluble wheat wastes [22]. By day 174, the abundance of *Megasphaera* increased notably; the main species detected—*Megasphaera cerevisiae*—is a lactate utilizer that produces acetic, butyric, caproic, and isovaleric acids [23]. *Megasphaera* could be an opportunistic species consuming lactate provided by *Lactobacillus*, which explains the absence of this metabolite in the VFA detected in the supernatant (Fig. 3b). Finally, several Lactobacillus spp. were present at all fermentation stages, e.g*., Lactobacillus sp., Lactobacillus rapi, Lactobacillus nagelii, Lactobacillus vini, Lactobacillus cassei, Lactobacillus plantarum*, and *Lactobacillus vaccinostercus*. The phyllosphere—the aerial part of living plants recognized as habitat for microorganisms— is colonized with lactic acid bacteria being a reservoir for these microorganisms in industrial and traditional fermentations [24]. Some Lactobacillus spp. are starter bacteria in these fermentations due to their rapid growth, which depends on the availability of sugar and protein found in complex media such as some plant materials. *Lactobacillus* spp. are well adapted to various niches, including fermentations due to their optimal nutrient utilization, presence of genes associated with acid resistance, and biofilm formation. The role of *Lactobacillus* in hydrogen-producing reactors has not yet been completely elucidated; some authors attribute the decline in productivity to the presence of *Lactobacillus* spp. [25], but other authors report highly productive processes based on cooperative interactions between Lactobacillus with lactate- and acetate-consumers via cross-feeding interactions [26]. In this study, *Lactobacillus* was the pioneer bacteria most abundant, possibly preparing the niche for *Enterococcus* and *Prevotella* to grow. The absence of lactic acid in the acidogenesis reactor also suggests cross-feeding interactions between *Lactobacillus* with lactate- and acetate-consumers.

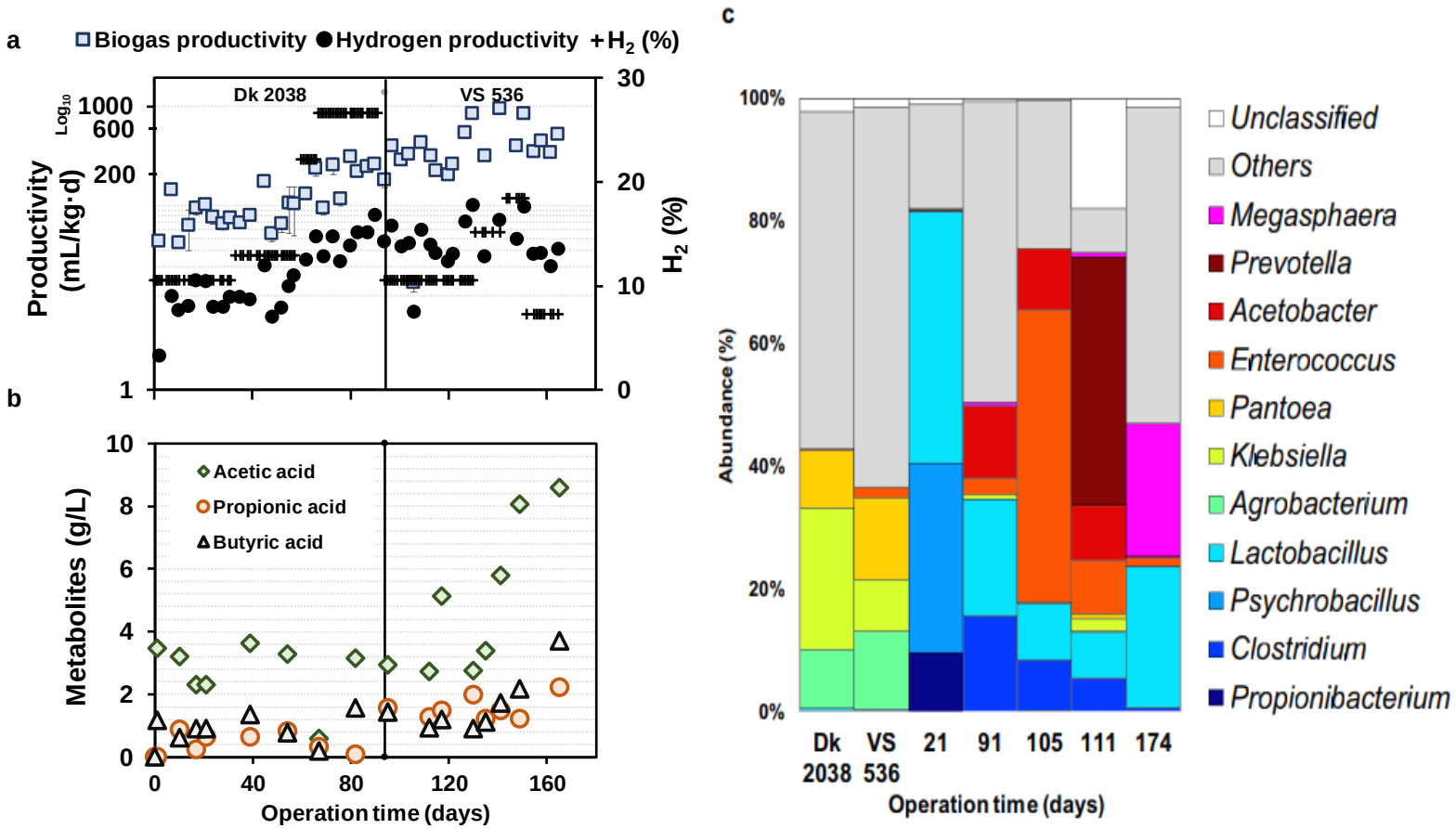

Fig. 3.1 (a) Performance of direct acidogenesis of raw corn stover for producing (a) hydrogen and (b) volatile fatty acids. (c) Changes in the bacterial microbiota during the acidogenesis of raw corn stove

3.4.2 ABE fermentation from spent solids of corn stover and VFA

The acidogenesis process left spent solids saturated with acetic, propionic, and butyric acids, some of which could serve as precursors of ABE fermentation [27]. Thus, a simplex centroid mixture design was used to elucidate the effects of acetic (X_1), propionic (X_2), and butyric (X_3) acids on the production of acetone (y_1), ethanol (y_2), and butanol (y_3) in a mixed culture at pH 6.5 and 7.5 (Table 3). Table 3 shows the respective coefficients of regression and ANOVA of the mathematical models adjusted to the response function. Generally, the mixed culture produced more ethanol than acetone and butanol, independent of pH (Table 3.1).

Table 3.1. Models, R^2, adjusted R^2, and probability values for the final reduced models (component proportion) of acids.

	pH 6.5	**pH 7.5**
Model	Cubic	Cubic
F calculated	20.61	30.63
F tabulated	79.10	111.54
R^2	0.9375	0.9608
Adjusted R^2	0.8920	0.9294
p-Value	< 0.0001	< 0.0001
Equation	y_3 = 0.029* X_1+4.169E-004* X_2+0.085* C-0.056* X_1 * B+0.013* X_1 * X_3-0.011* X_2 * C-0.075* X_1 * X_2 * X_3-0.095* X_1 * X_2 * (X_1- X_2)+0.13* X_1 * X_3 * (X_1- X_3)	y_3 = 9.899 E-005* X_1+9.854 E-005* X_2+0.12* X_3+4.087E-004* X_1 * X_2+0.014* X_1 * X_3+0.012* X_2 * X_3-0.70* X_1 * X_2 * X_3-0.40* X_1 * X_2 * (X_1- X_2) +0.24* X_1 * X_3 * (X_1- X_3)

Additionally, higher ethanol concentrations and the pair acetone/butanol were observed with different compositions of VFA. The higher ethanol concentrations were promoted by VFA mixtures with a higher ratio of acetic acid, mainly at pH 6.5 (Fig. 3.2). Higher concentrations of the pair acetone/ butanol were reached with VFA mixtures with a higher ratio of butyric acid.

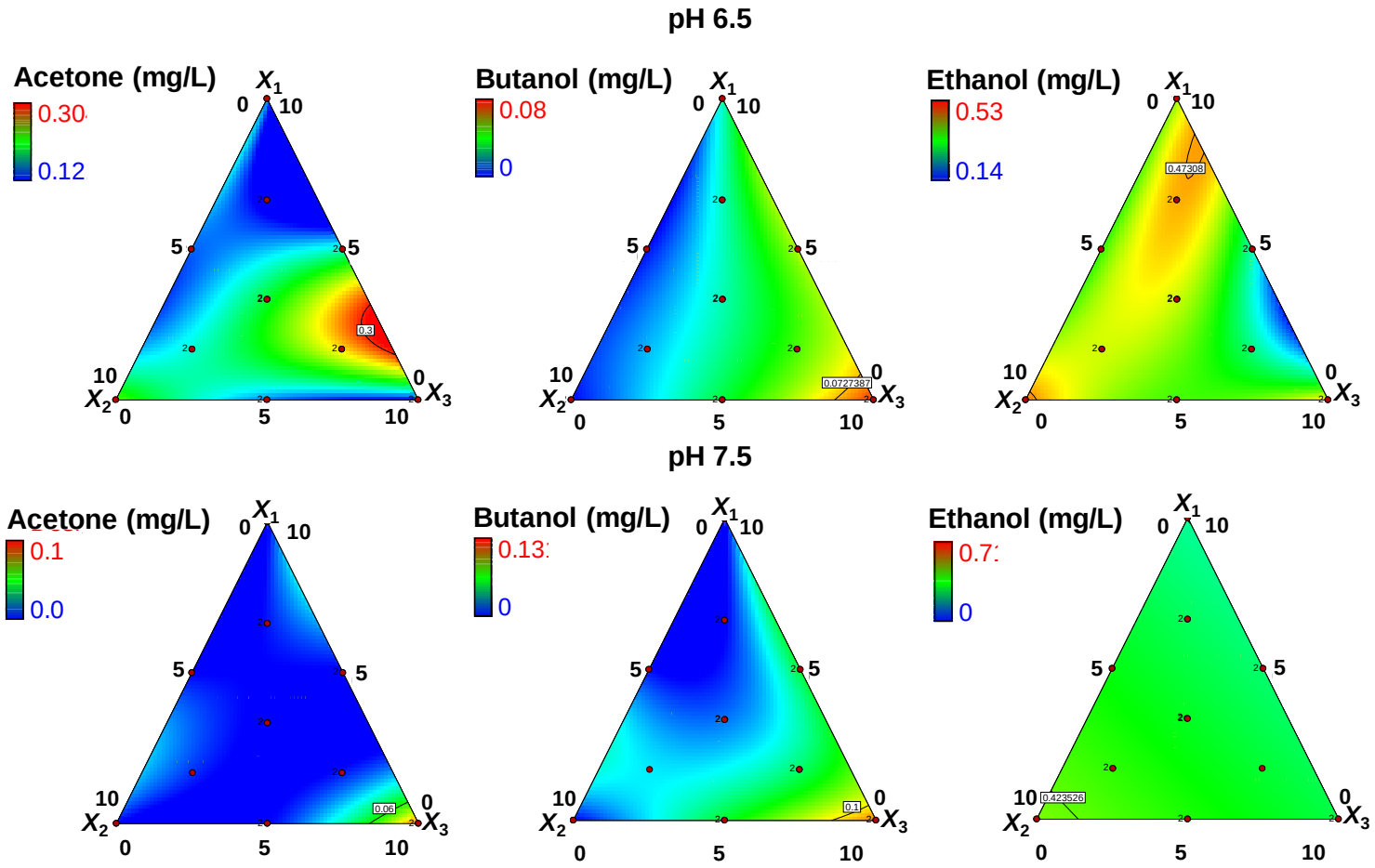

Fig. 3.2. Mixture contours plot interaction effects of VFA mixtures on the production of acetone, butanol, and ethanol. X_1, acetic acid (g/L); X_2, propionic acid (g/L); X_3, butyric acid (g/L).

According to the linear terms, pure butyric acid resulted in the highest butanol concentrations at pH 7.5 (Table 3.1 and 3.2), followed by the VFA mixtures with a higher ratio of butyric acid. Importantly, the mixed culture did not produce butanol in the absence of butyric acid (control and treatments B and F). Additionally, pure acetic and propionic acids or when these acids were in a higher ratio in the mixtures. Inhibited the production of butanol (treatments A, B, and H). The effects of acetic and butyric acids on the production of butanol differ among studies. However, most authors have reported that supplementation with butyric acid increases the concentration of butanol [28]; however, null results have also been observed [29]. In the present study, the mixed culture produced butanol only in the presence of butyric acid; otherwise, only ethanol was produced, similar to other studies [30]. In agreement with this study, Zhou et al. [31] reported that 10 g/L acetic acid completely inhibited butanol production. The same behavior was observed in treatments A and H, where pure acetic acid or mixtures with a higher ratio of acetic acid inhibited butanol production. The VFA mixtures affected butanol production, and the addition of pure butyric acid resulted in the highest butanol concentration of 120 mg/L. This butanol concentration is similar to that previously reported for a microbial consortium fermenting Napier grass directly as a substrate [32].

3.3.2.4 Procedure and experimental design

The treatments described in Section 3.3.2.2 selected to be bioaugmented with *C. saccharobutylicum* were the pure component of butyric acid (treatment C), the binary mixture of acetic and butyric acids (treatment E), the mixture with a higher ratio of butyric acid (treatment J), and an additional mixture with a similar ratio of those acids produced in the acidogenesis reactor (3.3, 0.8, 0.8). The control treatment consisted of a bottle without VFA supplementation. The reactors were loaded as previously was described in Section 3.2.2.1 (the nutrient solution was reduced to 100.8 mL to be supplemented with the single culture). The bottles were incubated at 37 °C for 1 day for consumption of oxygen by facultative bacteria present in the inoculum. Then, those reactors with bioaugmentation received 56.7 mL of the single culture of *C. saccharobutylicum,* meanwhile, those reactors without bioaugmentation received the same bacterial culture sterilized by autoclave. All treatments were performed in duplicate. Finally, the bottles were incubated at 37 °C for 6 days.

For improving the butanol titer, some treatments were bioaugmented with *C. saccharobutylicum*, and satisfactory results were obtained for all treatments; the concentration of butanol increased from 120 to 601 mg/L with the supplementation with pure butyric acid (Fig.

3.3). Previous authors have also increase butanol production to 3.7 g/L via bioaugmentation of a microbial consortium called N3 with the solventogenic species *Clostridium acetobutylicum* ATCC824 using filter paper (cellulose only) as the sole carbon source [33]. Although bioaugmentation improved the butanol titer, there were other factors at play, including inoculation time, initial concentration of inoculant, and inoculation ratio, which could be optimized to obtain economically competitive butanol titers.

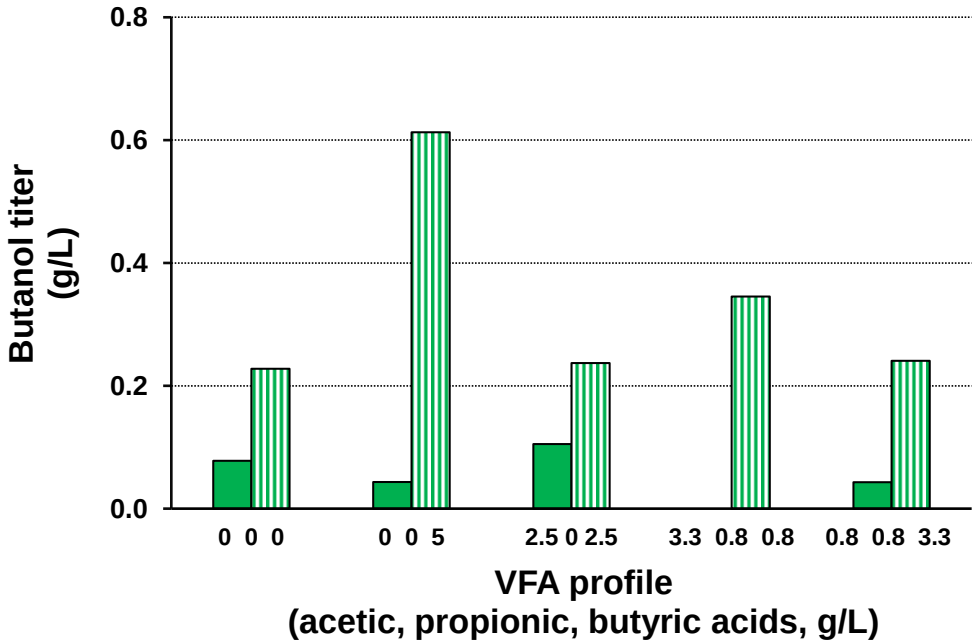

Fig. 3.3 Butanol production from spent solids of corn stover and different VFA profiles using a mixed culture with and without bioaugmentation with *Clostridium saccharobutylicum*

3.4.3 Butanol production from H_2/CO_2

Early studies have used mixtures of CO_2 and H_2 to produce carboxylic acids but not butanol [34–36]. In this study, three inocula were utilized to convert these gases to butanol using two medium formulations. All inocula performed poorly during the first period of operation, with butanol titers between 90 and 170 mg/L (Fig. 3.4a). During this period, inocula had a low CO_2 consumption rate without fixing all the CO_2 injected. The supernatant inoculum had the highest CO_2 consumption rate of 3.6 mmol/day. As a negative result, all inocula produced methane (from 1 to 17%). Then, the CO_2/H_2 gas mixture was suspended by day 110, and feeding was resumed on day 147 using the culture medium "B." During this period, sludge inoculum

produced the highest butanol concentration of 889 mg/L by day 196 (Fig. 3.4a). Supernatant and digestate inocula produced 180 and 380 mg/L butanol, respectively. Interestingly, in this second period, all inocula consumed all CO_2, between 10 and 24 days. The most notable was the supernatant inoculum, which reached the highest consumption rate of CO_2. The percentages of methane in the headspace ranged from 50 to 60% as the lowest percentages and up to 80%. Based on ANOVA, the type of inoculum and culture medium had a significant effect on butanol production ($p < 0.05$). By using the culture medium "B," all inocula behaved better in terms of the consumption rate of CO_2 and butanol production, which could be attributed to the trace metals contained in the culture medium "B" that had a positive effect on butanol production with pure cultures of Clostridium carboxidivorans fermenting syngas [9]. In this study, the maximum butanol concentration in the sludge inoculum reached 889 mg/L, a value similar to that previously reported using a pure culture of *Clostridium carboxidivorans*, which produced 1090 mg/L butanol from syngas [9]. Regarding the use of mixed culture, the butanol production with the sludge inoculum was 1.5-fold higher than that observed in batch fermentation of syngas (CO: CO_2: H_2: N_2 [20:20:10:50]) with a mixed culture previously acclimated to CO gas fermentation [12]. On the other hand, the maximum butanol concentration in this work was similar to that detected in syngas fermentation supplemented with 1.5 g/L butyric acid to improve butanol production with a mixed culture acclimatized to syngas fermentation (800 mg/L) [37].

For every inoculum tested, the time to reach the maximum production was higher than that detected in previous studies, mostly due to the use of a mixed culture that was not acclimatized to gas fermentation. It is important to remark that this previous study used syngas—a CO-rich stream—as the substrate, while this study is the first attempt to produce butanol from CO_2/H_2. The nutrients improved butanol production; therefore, optimizing the culture medium as well as the operating conditions is the next step to increase and stabilize the butanol concentrations from CO_2/H_2 using mixed culture. The mixed cultures were composed of non clostridial autotrophs (Fig. 3.4b). There was no pattern in the bacterial structure; Proteobacteria dominated in digestate and supernatant inocula, which shared the same origin, while sludge inoculum exhibited a different diversity structure. Members of these autotrophic communities have been previously identified in autotrophic environments. For instance, Pseudomonas contains a great deal of metabolic diversity, where *Pseudomonas oxalaticus* performs autotrophic CO_2 fixation via the Calvin cycle. A previous study characterizeda synthetic

autotrophic consortium composed of *Pseudomonas, Ochrobactrum*, and *Stenotrophomonas* [38], three genera found in this study. In that study, the authors identified *Ochrobactrum* as the main species responsible for CO_2 fixation via the Calvin-Benson-Bassham pathway, while Pseudomonas had a heterotrophic metabolism, reducing the inhibition of extracellular metabolites from autotrophic microorganisms, and *Stenotrophomonas* improved the carbon fixation efficiency. *Dechloromonas* was described as a hydrogenoxidizing bacterium that grows with CO_2 as the sole carbon source and isolated from a gas-phase anaerobic packed bed biofilm reactor treating perchlorate-contaminated groundwater [39]. *Dechloromonas* also grew with acetate as an electron donor. *Janthinobacterium* is a heterotrophic aerobic bacterium with capnophilic behavior, which means that this genus grows in CO_2 atmospheres. This genus possesses the same enzymes present in methanogens that reduce CO_2 to methane, and these enzymes fix CO_2 via dark Crassulacean acid metabolism [40]. Based on the literature review, it is feasible that the members of autotrophic mixed cultures perform different tasks inside gas fermentation reactors, including fixation of CO_2, reduction of CO_2 to methane, and consumption of VFA. However, with the available data, it is not possible to identify which genera were directly involved in butanol production, and further studies are required. The structures of the autotrophic mixed cultures performing the fermentation of H_2 and CO_2 in this study differed from those of communities fermenting syngas previously reported. These differences could be explained by the absence of CO in the gaseous substrate used here. Carboxydotrophi bacteria such as *Clostridium carboxidivorans* are known to catalyze the oxidation of CO to CO_2 using the electrons derived for growth and production of soluble metabolites [37]. In contrast, the off-gas from the acidogenesis reactor contains only H_2 and CO_2, not CO; consequently, a variety of microbial communities thrived in such conditions, and the ability of these communities to fix CO_2 must be explored.

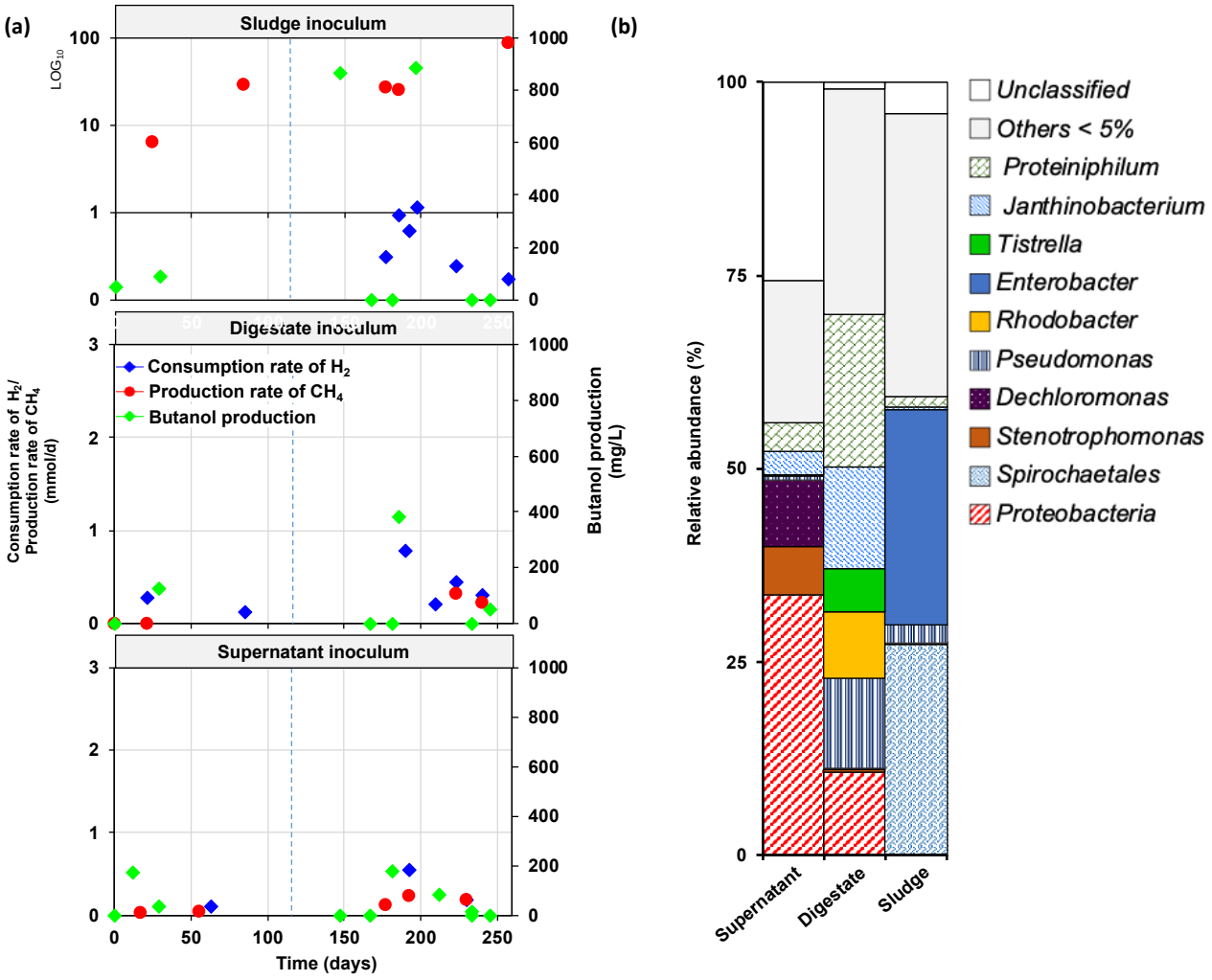

Fig. 3.4 a. Performance of gas fermentation of CO_2/H_2 for producing butanol. b. Diversity of autotrophic mixed cultures fermenting CO_2/H_2.

3.4.4 A cascading process for improved carbon utilization

Few studies have described processes to convert raw lignocellulosic substrates to biofuels and chemicals. Here, a cascading process that integrates two consolidated bioprocesses with CO_2 fermentation was presented (Fig. 3.5). In this study, each stage was studied independently of the others, although some output flows served for feeding the following process. The first unit of acidogenesis operated as a consolidated bioprocess hydrolyzing and fermenting the raw substrate for more than 6 months, and the productivities of hydrogen and VFA tended to increase. The conversion of raw corn stover to VFA was 38.2 g C/100 g C, mainly acetic acid followed by butyric and propionic acids. The gas composition was enriched mainly with CO_2 (3.1 g C/100 g C) with no more than 27% hydrogen (acidogenesis reactor, Fig. 3.5). Then, digestates (spent solids) of the acidogenesis stage served to produce butanol in a second consolidated bioprocess. Since the aim was to evaluate the effects of different compositions of VFA on ABE fermentation, such digestates were washed and then supplemented with synthetic mixtures of VFAs. The results demonstrated that the VFA composition affected the ABE fermentation, where the addition of pure butyric acid improved the butanol titer almost three times in comparison with control (without the addition of butyric acid). Consequently, the successful coupling of acidogenesis with ABE fermentation depended severely on the VFA composition produced in the acidogenesis stage. The carbon conversion of spent solids to butanol was approximately 2 g C/100 g C (ABE fermentation, Fig. 3.5). In literature, there are few studies of consolidated bioprocesses devoted to butanol production. Carbon conversion for pure cultures of Clostridium spp. fermenting cellulose or treated lignocellulose materials to butanol ranged from 15 to 21% (butanol titers from 1.4 to 14 g/L, [41–44]. Although, other studies have reported carbon conversions similar to that obtained in this study of 2% (butanol titer 0.35 g/L) [45, 46]. Some fermentation parameters could have influenced the butanol titer in this study. The ABE products were measured after 6 days of incubation, this incubation time could be lower than that required time to observe the highest butanol production, for example, Yang et al. [43] required 10 days of incubation to observe the highest butanol titer from cellulose. Also, the solventogenic species found in the mixed culture may require further acclimatization or genetic manipulation to overcome the toxicity of butanol [47]. Besides, as mentioned earlier, the inoculation timing and ratio of inoculants are determinant parameters in the bioaugmentation strategy that should be studied [42]. Finally, the third process fixed CO_2 to butanol and other VFA. In combination with an adequate medium formulation, the sludge

inoculum converted 36% of carbon to soluble products, of which 73% was butanol (gas fermentation, Fig. 3.5). The acclimatization of this inoculum occurred using the model gas CO_2/ H_2 with a composition of 75:25 (Fig. 3.5). In contrast, the acidogenesis stage produced H_2/CO_2 with a composition of 73:27. This gas composition likely affected butanol production, and this hypothesis requires further research. Thus, the gas composition is the key factor determining the successful coupling of acidogenesis with CO_2 fermentation using continuous gas fermentation reactors. A drawback of gas fermentation with mixed cultures is the undesired generation of methane—a more potent greenhouse gas than CO_2—requiring further studies to eliminate its presence.

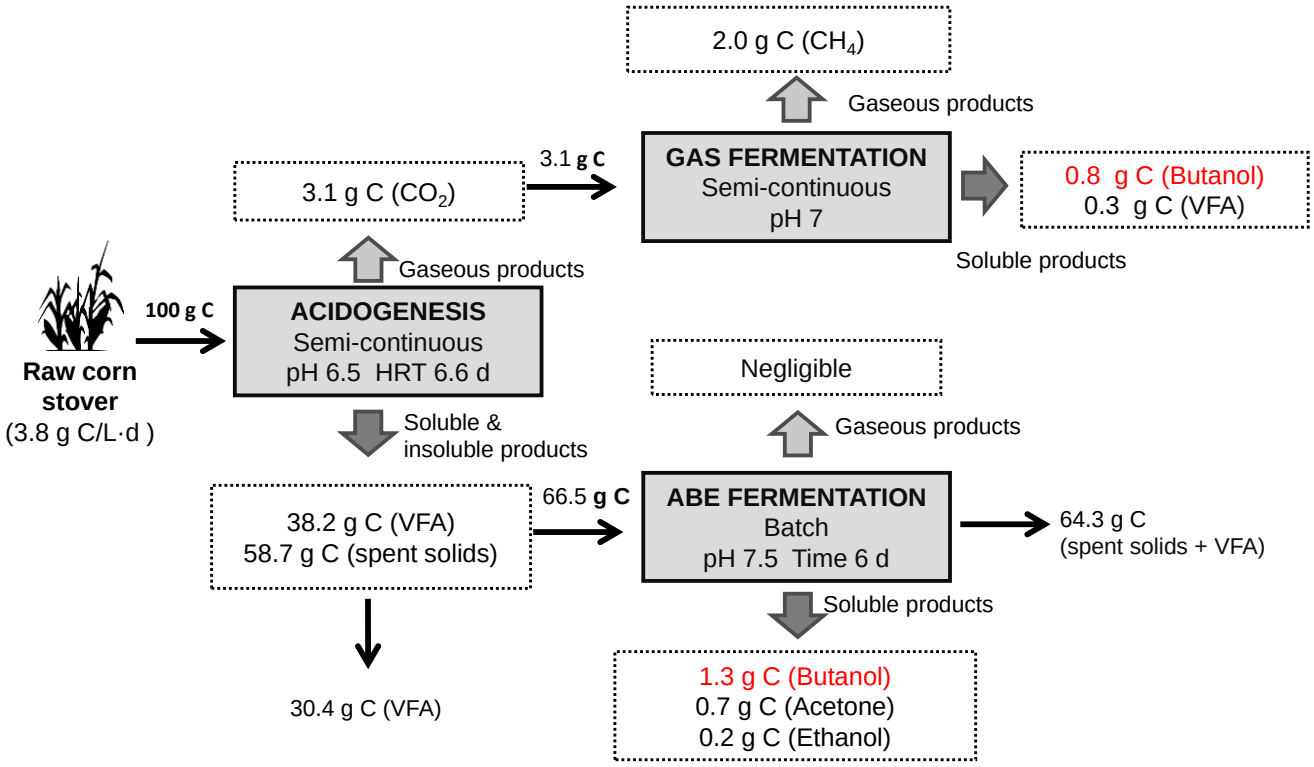

Fig. 3.5 Carbon balance for a cascade process that produces butanol from raw corn stover

3.5 Conclusions

This study presents for the first time the experimental results of the integration of direct acidogenesis of a lignocellulosic substrate with ABE and gas fermentations using mixed cultures. The acidogenesis process was stable during 195 days with maximum hydrogen productivity of 87 mL/L day and a total VFA production of up to 15 g/L. The spontaneous acidogenesis of corn stover resulted in an ecological succession where *Prevotella* and

Megasphaera colonized the lignocellulosic substrate at the later stages of fermentation. Then, the spent solids of corn stover served as substrate, and 230 mg/L butanol was produced using a mixed culture bioaugmented with *C. saccharobutylicum*. The addition of butyric acid was a key factor in improving ABE fermentation, and a butanol titer of 610 mg/L was reached. In contrast, acetic and propionic acids inhibited ABE fermentation. Finally, an autotrophic mixed culture composed mainly of *Spirochaetales, Enterobacter*, and *Pseudomonas* converted CO_2 to butanol with a maximum titer of 889 mg/L.

3.6 References

1. Ren NQ, Zhao L, Chen C, Guo WQ, Cao GL (2016) A review on bioconversion of lignocellulosic biomass to H2: key challenges and new insights. Bioresour Technol 215:92–99. https://doi.org/10.1016/j.biortech.2016.03.124
2. Bharathiraja B, Jayamuthunagai J, Sudharsanaa T, Bharghavi A, Praveenkumar R, Chakravarthy M, Yuvaraj D (2017) Biobutanol- an impending biofuel for future: a review on upstream and downstream processing techniques. Renew Sustain Energy Rev 68:788–807. https://doi.org/10.1016/j.rser.2016.10.017
3. Li J, Chi X, Zhang Y, Wang X (2018) Enhanced coproduction of hydrogen and butanol from rice straw by a novel two-stage fermentation process. Int Biodeterior Biodegrad 127:62–68. https://doi.org/10.1016/j.ibiod.2017.11.004
4. Valdez-Vazquez I, Alatriste-Mondragón F, Arreola-Vargas J, Buitrón G, Carrillo-Reyes J, León-Becerril E, Mendez-Acosta HO, Ortíz I, Weber B (2020) A comparison of biological, enzymatic, chemical and hydrothermal pretreatments for producing biomethane from Agave bagasse. Ind Crops Prod 145:112160. https://doi.org/10.1016/j.indcrop.2020.112160
5. Atasoy M, Owusu-Agyeman I, Plaza E, Cetecioglu Z (2018) Biobased volatile fatty acid production and recovery from waste streams: current status and future challenges. Bioresour Technol 268:773–786. https://doi.org/10.1016/j.biortech.2018.07.042
6. Chu Y, Wei Y, Yuan X, Shi X (2011) Bioconversion of wheat stalk to hydrogen by dark fermentation: effect of different mixed microflora on hydrogen yield and cellulose solubilisation. Bioresour Technol 102(4):3805–3809. https://doi.org/10.1016/j.biortech.2010.11.092
7. Sanchez A, Magaña G, Gomez D, Solís M, Banares-Alcantara R (2014) Bidimensional sustainability analysis of lignocellulosic ethanol production processes. Method and case

study. Biofuel Bioprod Bioref 8(5):670–685. https://doi.org/10.1002/bbb.1512

8. Müller V (2019) New horizons in acetogenic conversion of one carbon substrates and biological hydrogen storage. Trends Biotechnol 37(12):1344–1354. https://doi.org/10.1016/j.tibtech.2019.05.008
9. Phillips JR, Atiyeh HK, Tanner RS, Torres JR, Saxena J, Wilkins MR, Huhnke RL (2015) Butanol and hexanol production in Clostridium carboxidivorans syngas fermentation: medium development and culture techniques. Bioresour Technol 190:114–121. https://doi.org/10.1016/j.biortech.2015.04.043
10. Shen S, Gu Y, Chai C, Jiang W, Zhuang Y, Wang Y (2017) Enhanced alcohol titre and ratio in carbon monoxide-rich off-gas fermentation of Clostridium carboxidivorans through combination of trace metals optimization with variable-temperature cultivation. Bioresour Technol 239:236–243. https://doi.org/10.1016/j.biortech.2017.04.099
11. Ramió-Pujol S, Ganigué R, Bañeras L, Colprim J (2015) Incubation at 25 °C prevents acid crash and enhances alcohol production in Clostridium carboxidivorans P7. Bioresour Technol 192:296–303. https://doi.org/10.1016/j.biortech.2015.05.077
12. Chakraborty S, Rene ER, Lens PLN, Veiga MC, Kennes C (2019) Enrichment of a solventogenic anaerobic sludge converting carbon monoxide and syngas into acids and alcohols. Bioresour Technol 272:130–136. https://doi.org/10.1016/j.biortech.2018.10.002
13. van Soest PJ (1990) Use of detergents in the analysis of fibrous feeds I. Preparation of fiber residues of low nitrogen content. Journal of Association of Official Analytical Chemists 73(4):487–491. https://doi.org/10.1093/jaoac/73.4.487
14. APHA Standard methods for the examination of water and wastewater, 20th edition. American Public Health Association, 1999. Washington DC.
15. Pérez-Rangel M, Quiroz-Figueroa FR, González-Castañeda J, Valdez-Vazquez I (2015) Microscopic analysis of wheat straw cell wall degradation by microbial consortia for hydrogen production. Int J Hydrog Energy 40(1):151–160. https://doi.org/10.1016/j.ijhydene.2014.10.050
16. Valdez-Vazquez I, Morales AL, Escalante AE (2017) History of adaptation determines short-term shifts in performance and community structure of hydrogen-producing microbial communities degrading wheat straw. Microb Biotechnol 10(6):1569–1580. https://doi.org/10.1111/1751-7915.12678
17. Valdez-Vazquez I, Poggi-Varaldo MH (2009) Hydrogen production by fermentative

consortia. Renew Sustain Energy Rev 13(5): 1000–1013. https://doi.org/10.1016/j.rser.2008.03.003

18. Muñoz-Páez KM, Alvarado-Michi EL, Moreno-Andrade I, Buitrón G, Valdez-Vazquez I (2020) Comparison of suspended and granular cell anaerobic bioreactors for hydrogen production from acid agave bagasse hydrolyzates. Int J Hydrog Energy 45(1):275–285. https://doi.org/10.1016/j.ijhydene.2019.10.232
19. Rachbauer L, Beyer R, Bochmann G, Fuchs W (2017) Characteristics of adapted hydrogenotrophic community during biomethanation. Sci Total Environ 595:912–919. https://doi.org/ 10.1016/j.scitotenv.2017.03.074
20. Wolfe BE, Dutton RJ (2015) Fermented foods as experimentally tractable microbial ecosystems. Cell 161(1):49–55. https://doi.org/ 10.1016/j.cell.2015.02.034 Biomass Conv. Bioref.
21. Vorholt JA (2012) Microbial life in the phyllosphere. Nat Rev Microbiol 10:828. https://doi.org/10.1038/nrmicro2910
22. De Paepe K, Verspreet J, Courtin CM, Van de Wiele T (2020) Microbial succession during wheat bran fermentation and colonisation by human faecal microbiota as a result of niche diversification. ISME J 14:584–596. https://doi.org/10.1038/s41396-019-0550-5
23. Engelmann U, Weiss N (1985) Megasphaera cerevisiae sp. nov.: a new gram-negative obligately anaerobic coccus isolated from spoiled beer. System Appl Microbiol 6:287–290
24. Douillard FP, de Vos WM (2014) Functional genomics of lactic acid bacteria: from food to health. Microb Cell Fact 13: S8. https://doi.org/10.1186/1475-2859-13-S1-S8
25. Castelló E, Ferraz-Junior ADN, Andreani C, Anzola-Rojas MP, Borzacconi L, Buitrón G, Carrillo-Reyes J, Damasceno Gomes S, Maintinguer SI, Moreno-Andrade I, Palomo-Briones R, RazoFlores E, Schiappacasse Dasati M, Tapia-Venegas E, ValdezVazquez I, Vesga-Baron A, Zaiat M, Etchebehere C (2019) Stability problems in the hydrogen production by dark fermentation: possible causes and solutions. Renew Sustain Energy Rev 119:109602. https://doi.org/10.1016/j.rser.2019.109602
26. García-Depraect O, Díaz-Cruces VF, Rene ER, León-Becerril E (2019) Changes in performance and bacterial communities in a continuous biohydrogen-producing reactor subjected to substrateand pH-induced perturbations. Bioresour Technol 295:122182. https://doi.org/10.1016/j.biortech.2019.122182
27. Molina-Guerrero CE, Valdez-Vazquez I, Sánchez A, Vázquez Castillo JA, Vazquez-Nuñez

E (2020) A biorefinery based on the biomechanical configuration of the digestive system of a ruminant for ABE production: a consolidated bioprocessing approach. Biomass Convers Biorefin. https://doi.org/10.1007/s13399-020-00620-5

28. Zhou Q, Liu Y, Yuan W (2020) Kinetic modeling of butyric acid effects on butanol fermentation by Clostridium saccharoperbutylacetonicum. New Biotechnol 55:118–126. https://doi.org/10.1016/j.nbt.2019.10.004
29. Al-Shorgani NKN, Kalil MS, Yusoff WMW, Hamid AA (2018) Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of Clostridium acetobutylicum YM1. Saudi J Biol Sci 25(2):339–348.https://doi.org/10.1016/j.sjbs.2017.03.020
30. De Souza MB, Mary dos Santos G, Palladino Delforno T, Tadeu Fuess L, José da Silva A (2019) Enriched microbial consortia for dark fermentation of sugarcane vinasse towards value-added shortchain organic acids and alcohol production. J Biosci Bioeng 127(5):594–601. https://doi.org/10.1016/j.jbiosc.2018.10.008
31. Zhou Q, Liu Y, Yuan W (2018) Kinetic modeling of lactic acid and acetic acid effects on butanol fermentation by Clostridium saccharoperbutylacetonicum. Fuel 226:181–189. https://doi.org/10.1016/j.fuel.2018.04.019
32. Lin C-W, Wu C-H, Tran D-T, Shih M-C, Li W-H, Wu C-F (2011) Mixed culture fermentation from lignocellulosic materials using thermophilic lignocellulose-degrading anaerobes. Process Biochem 46(2):489–493. https://doi.org/10.1016/j.procbio.2010.09.024
33. Wang Z, Cao G, Zheng J, Fu D, Song J, Zhang J, Zhao L, Yang Q (2015) Developing a mesophilic co-culture for direct conversion of cellulose to butanol in consolidated bioprocess. Biotechnol Biofuels 8:84. https://doi.org/10.1186/s13068-015-0266-3
34. Arslan D, Steinbusch KJJ, Diels L, De Wever H, Buisman CJN, Hamelers HVM (2012) Effect of hydrogen and carbon dioxide on carboxylic acids patterns in mixed culture fermentation. Bioresour Technol 118:227–234. https://doi.org/10.1016/j.biortech.2012.05.003
35. Arslan D, Steinbusch KJJ, Diels L, De Wever H, Hamelers HVM, Buisman CJN (2013) Selective carboxylate production by controlling hydrogen, carbon dioxide and substrate concentrations in mixed culture fermentation. Bioresour Technol 136:452–460. https://doi.org/10.1016/j.biortech.2013.03.063
36. Darvekar P, Liang C, Nazmul Karim M, Holtzapple MT (2019) Effect of headspace gas

composition on carboxylates production in open culture fermentation of corn stover. Biomass Bioenergy 126:57–61. https://doi.org/10.1016/j.biombioe.2019.04.019

37. Liu K, Atiyeh HK, Stevenson BS, Tanner RS, Wilkins MR, Huhnke RL (2014) Mixed culture syngas fermentation and conversion of carboxylic acids into alcohols. Bioresour Technol 152:337–346. https://doi.org/10.1016/j.biortech.2013.11.015
38. Hu J, Xue Y, Li J, Wang L, Zhang S, Wang Y, Gao M (2016) Characterization of a designed synthetic autotrophic–heterotrophic consortia for fixing CO2 without light. RSC Adv 6:78161–78169. https://doi.org/10.1039/C6RA13118B
39. Zhang H, Bruns MA, Logan BE (2002) Perchlorate reduction by a novel chemolithoautotrophic, hydrogen-oxidizing bacterium. Environ Microbiol 4(10):570–576. https://doi.org/10.1046/j.1462-2920.2002.00338.x
40. Valdes N, Soto P, Cottet L, Alarcon P, Gonzalez A, Castillo A, Corsini G, Tello M (2015) Draft genome sequence of Janthinobacterium lividum strain MTR reveals its mechanism of capnophilic behavior. Stand Genomic Sci 10:110. https://doi.org/10.1186/s40793-015-0104-z
41. Nakayama S, Bando Y, Ohnishi A, Kadokura T, Nakazato A (2013) Decreased hydrogen production leads to selective butanol production in co-cultures of Clostridium thermocellum and Clostridium saccharoperbutylacetonicum strain N1-4. J. Biosci Bioeng 115(2): 173–175. https://doi.org/10.1016/j.jbiosc.2012.08.020
42. Wen Z, Wu M, Lin Y, Yang L, Lin J, Cen P (2014) Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of Clostridiumbeijerinckii and Clostridium cellulovorans. Microb Cell Fact 13(1): 92. https://doi.org/10.1186/s12934-014-0092-5
43. Yang X, Xu M, Yang ST (2015) Metabolic and process engineering of Clostridium cellulovorans for biofuel production from cellulose. Metab Eng 32:39–48. https://doi.org/10.1016/j.ymben.2015.09.001
44. Valdez-Vazquez I, Pérez-Rangel M, Tapia A, Buitrón G, Molina CE, Hernández G, Amaya-Delgado L (2015) Hydrogen and butanol production from native wheat straw by synthetic microbial consortia integrated by species of Enterococcus and Clostridium. 159: 214-222. https://doi.org/10.1016/j.fuel.2015.06.052
45. Salimi F, Mahadevan R (2013) Characterizing metabolic interactions in a clostridial co-culture for consolidated bioprocessing. BMC Biotechnology 13(1):95.

https://doi.org/10.1186/1472-6750-13-95
46. Tian L, Conway PM, Cervenka ND, Cui J, Maloney M, Olson DG, Lynd LR (2019) Metabolic engineering of Clostridium thermocellum for n-butanol production from cellulose. Biotechnol Biofuels 12:186. https://doi.org/10.1186/s13068-019-1524-6
47. Chen BY, Chuang FY, Lin CL, Chang JS (2012) Deciphering butanol inhibition to Clostridial species in acclimatized sludge for improving biobutanol production. Biochem Eng J 69:100–105. https://doi.org/10.1016/j.bej.2012.09.005

Capítulo 4.0 Resultados. Evolución adaptativa de laboratorio aplicada a una comunidad microbiana acidógena para mejorar la producción de butanol a partir de celulosa de rastrojo de maíz

El capítulo 4 estuvo dedicado al cumplimiento del objetivo específico 3, la segunda estrategia para incrementar la concentración del butanol en la fermentación ABE, en este caso de optó por aplicar la evolución adaptativa en laboratorio (EAL).

De los resultados del objetivo 2 sobre la fermentación ABE (Capítulo 3), se encontró que aún con la bioaumentación, el consorcio microbiano produjo una concentración no satisfactoria de butanol, < 1 g/L. Por tanto, la motivación de este trabajo fue aplicar la EAL para obtener un consorcio con tolerancia al butanol. Por estudios anteriores, se sabe que el butanol es tóxico para la mayoría de las células a concentraciones mayores a 2 g/L, ya que al ser liposoluble incrementa la fluidez de la membrana plasmática e interfiere con los procesos de transporte de nutrientes. La EAL ha sido una estrategia utilizada en cultivos puros incrementando la tolerancia a un agente estresor. La EAL consistió en someter a un consorcio microbiano acidogénico a cuatro concentraciones iniciales de butanol (0.25, 0.50, 1.0, y 2.0 g/L) que fueron incrementando a razón de 0.25 g/L durante seis incubaciones de siete días cada una. Después, los consorcios microbianos fueron sometidos a una concentración de butanol de 5 g/L durante tres incubaciones. Se mantuvo el consorcio microbiano parental sin adición de butanol como control durante todo el experimento. Durante la EAL se utilizaron residuos de maíz tratados térmicamente como fuente de azúcares complejos.

Los resultados mostraron que la EAL fue exitosa en modificar el patrón de fermentación de los consorcios en todas las concentraciones de butanol probadas. De este modo, a diferencia del consorcio microbiano parental, los consorcios microbianos sometidos a butanol produjeron más butirato que acetato. Además, en estos consorcios se observó que el butanol adicionado no se acumuló durante las incubaciones, contrario a esto, la concentración de butanol disminuyó al final de cada fermentación. La adición de butanol hizo que el consorcio microbiano se enriqueciera en *Bacteroides*, *Caproiciproducens*, *Anaerofilium*, *Acetanaerobacterium*, una especie no identificada del Orden Clostridia UCG-014, y *Pseudomonas*. Este consorcio tuvo la

capacidad de producir hasta 13.8 g/L de butanol directamente a partir de los polisacáridos del rastrojo de maíz.

Estos resultados de producción de butanol sirvieron para desarrollar el objetivo específico 5, en la simulación de las biorrefinerías (Capítulo 6), en específico en la fermentación ABE.

Referencia del trabajo sometido para publicación

González-Tenorio D, Valdez-Vazquez I. Adaptive laboratory evolution applied to an acidogenic microbial community for enhanced butanol production from corn stover cellulose.

4.1 Abstract

Butanol production is carried out mainly by pure cultures while microbial consortia produce unsatisfactory concentrations. Adaptive laboratory evolution (ALE) has been successfully applied to pure cultures improving the butanol tolerance, and consequently their production. In this study, ALE consisted of subjecting to an acidogenic consortium to four initial butanol concentrations (0.25, 0.50, 1.0, and 2.0 g/L) that were increased at 0.25 g/L during six incubations of seven days each. Then, the resulting consortia were subjected to a butanol shock of 5 g/L twice. Corn stover cellulose was used as substrate during ALE experiments. ALE was successful in modifying the fermentation products and the microbial structure. In contrast to the parental consortium, the consortia that received butanol produced more butyrate than acetate. Furthermore, the butanol added did not accumulate during the incubations, instead, the butanol concentration decreased at the end of each incubation. The addition of butanol enriched the microbial consortia in *Bacteroides*, *Caproiciproducens*, *Anaerofilium*, *Acetanaerobacterium*, an unidentified species of the Order Clostridia UCG-014, and *Pseudomonas*. After twelve incubations, the microbial consortium adapted to the highest butanol concentration produced 13.8 g/L after 4 days of incubation, and then the butanol was catalyzed.

Keywords: Butanol tolerance, consolidated bioprocessing, *Pseudomonas*.

4.2 Introduction

Butanol is an attractive transportation fuel since it can be blended with gasoline or diesel at any percentage, it has a high-energy content and octane number, and its blends do not separate in the presence of water, among other advantages [1]. A promising way to produce renewable butanol is from lignocellulosic biomass derived from agricultural, forestry, or agro-industrial waste. Such biomass is characterized by having a high content of carbohydrates made up of cellulose (35-50%), hemicellulose (20-35%), and lignin (10.25%).

The biological production of butanol occurs via the acetone-butanol-ethanol (ABE) fermentation, a two-stage process: the acidogenesis stage where exponential-phase cells produce acetate, butyrate, hydrogen, and carbon dioxide, and then the solventogenesis stage where stationary-phase cells re-assimilate acetate and butyrate to produce ABE. In nature, bacteria of the *Clostridium* genus (mainly *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharoperbutylacetonicum*, and *Clostridium saccharobutylicum*) perform the ABE fermentation [2]. The main drawback of ABE fermentation is that product concentrations between 12 to 20 g/L inhibit cellular metabolism resulting in a low product yield. The butanol toxicity towards bacteria increases the membrane fluidity and alters the processes of transport and absorption of glucose [2, 3]. Those more tolerant microorganisms to butanol achieve a higher product yield since more cells remain active until the end of fermentation. Tools such as genetic or metabolic engineering have increased the butanol tolerance of microorganisms [4]. For example, *Clostridium acetobutylicum* JB200 is a hyper butanol tolerant and producing strain obtained from *Clostridium acetobutylicum* ATCC 55025 through mutagenesis and adaptation [5]. *C. acetobutylicum* JB200 produces 20.3 ± 1. 8 g/L, 1.6 times more than ATCC 55025.

Alternatively, adaptive laboratory evolution (ALE) uses the principles of natural evolution, although implemented in the laboratory under controlled conditions to select improved phenotypes with the desired characteristics [4, 6]. Diverse research studies applied ALE focused on the improvement of butanol tolerance of *Clostridium* spp., some co-cultures with *Clostridium*, *Saccharomyces cerevisiae*, and engineered *Escherichia coli* [4]. For example, *Clostridium acetobutylicum* T64 improved their tolerance and butanol production through ALE from 12.2 g/L to 15.3 g/L using corn flour as substrate [7]. Likewise, [8] et al. (2019) used *Clostridium cellulovorans* with inserted genes of *Clostridium acetobutylicum* ATCC 824 for

butanol production from alkaline hydrolyzates of corn cobs. Then, they improved the butanol tolerance through ALE, increasing the concentration from 0.025 to 3.47 g/L.

ALE has proven highly effective for improving butanol production using monocultures and co-cultures of two microorganisms. However, it remains unclear if ALE could improve the butanol tolerance and production by microbial consortia. The advantage of using microbial consortia for butanol production is that they work as a consolidated bioprocess, thus, enzyme production, saccharification and fermentation occur simultaneously. Until now, the butanol production by microbial consortia is insignificant, barely 230 mg/L [9]. Therefore, this work aims to apply ALE to an acidogenic microbial consortium for improving butanol production from corn stover cellulose by consolidated bioprocessing.

4.3 Methodology

The inoculum consisted of an indigenous microbial community of corn stover that carried out an acidogenesis process [9]. The indigenous microbial community was subjected to a heat treatment at 105 °C for 12 h to select *Clostridium* spores [10]. The substrate consisted of corn stover treated by steam explosion at 45 psig, 150°C for 40 min [11]. The chemical composition of substrate determined using an automated fiber analyzer (ANKOM 200 Fiber Analyzer, ANKOM Technologies, Macedon, NY) was as follows: 21% extractives, 41% cellulose, 30% hemicellulose, and 8% lignin. Before its use, the steam-exploded corn stover was washed once with distilled water to eliminate inhibitors and soluble sugars. The culture medium used for the assays had the following composition (per liter): 2 g of urea, 0.5 g of KH_2PO_4, 2.1 g of NaH_2PO_4, 0.2 g of $NiSO_4$, 0.2 g of Na_2SO_4, 0.2 g of $FeCl_3 \cdot 4H_2O$, 0.2 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $CaCl_2 \cdot 6H_2O$, 0.1 g of $ZnCl_2$, 0.02 g of $CoCl_2$, and 0.01g $NaMo_4 \cdot 2H_2O$ [10, 12].

4.3.1 Adaptive evolution experiment

4.3.1.1 Stage I (1^{st} – 6^{th} incubations)

ALE was carried out in 250 mL glass serum bottles (reactors) with a working volume of 100 mL. At the beginning, the reactors were loaded with 3.3 g of substrate, 6.7 g of dry inoculum and filled up to 100 mL with non-sterile culture medium. In this stage, four reactors were incubated with increasing butanol concentrations, starting at concentrations: 0.25 mg/L (treatment A), 0.50 mg/L (treatment B), 1.0 mg/L (treatment C), and 2.0 mg/L (treatment D), all concentrations below the toxic threshold [13]. A treatment without butanol (0 mg/L) served as the control. The initial pH was adjusted to 7.5 with NaOH 0.1 M. The reactors were hermetically closed with air

in the headspace and incubated statically at 37 °C for seven days (first incubation). In the following incubations (from second to sixth incubation each of seven days), 60 % of supernatant (without solids) was replaced with fresh culture medium with an increasing butanol concentration as suggested by [14] et al. (2012). Each treatment received a butanol increment of 0.25 mg/L, *e.g.*, for the treatment with an initial butanol concentration of 0.25 mg/L, the final concentration at the sixth incubation was 1.50 mg/L. Thus, the maximum butanol concentrations were: 1.50 mg/L, 1.75 mg/L, 2.25 mg/L, and 3.25 mg/L for treatments A, B, C and D, respectively (Figure 4.1). The control treatment received only a fresh culture medium. All treatments were performed in duplicate. The productions of acetate, butyrate, and butanol were analyzed on Day 7.

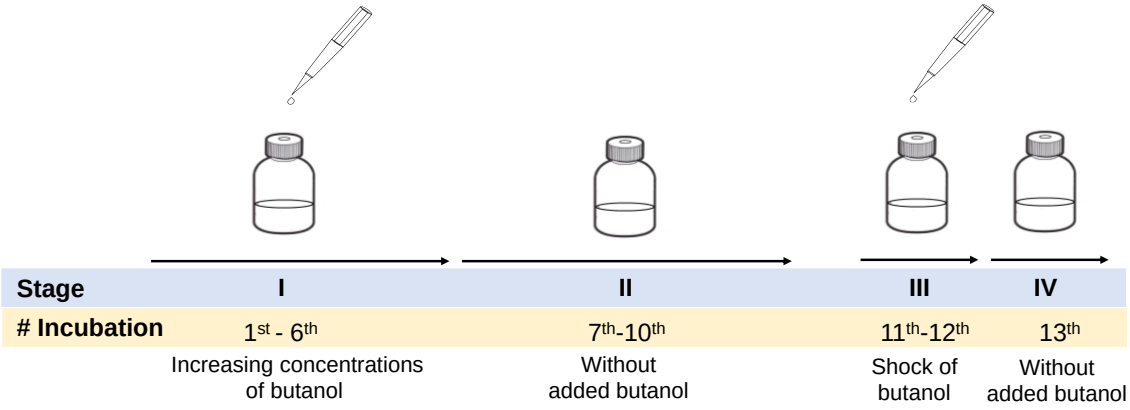

Figure 4.1 Adaptive laboratory evolution of an acidogenic microbial community for increased butanol production.

4.3.1.2 Stage II (7th to 10th incubations)

Concluded Stage I, 50% of supernatant was replaced with fresh culture medium without butanol, then, the reactors were incubated for seven days (seventh incubation). For the eighth incubation, 50% of supernatant was replaced one more time with fresh culture medium without butanol, and 60% of the fermented substrate (6 g) was replaced with fresh substrate and incubated for seven days. The procedure applied in the seventh and eighth incubations was repeated in the ninth and tenth incubations, respectively. The productions of acetate, butyrate, and butanol were analyzed on Day 7.

4.3.1.3 Stage III (11th and 12th incubations)

For the eleventh and twelfth incubations, 50% of supernatant was replaced with fresh culture medium, 60% of the fermented substrate was replaced with fresh substrate, and 5 g/L of butanol was added. After, the reactors were incubated for seven days every time. The productions of acetate, butyrate, and butanol were analyzed on Day 7.

4.3.1.4 Stage IV (13th incubation)

Finally, in the thirteenth incubation, 60% of the fermented substrate was replaced with fresh substrate, 50% of supernatant was replaced with fresh culture medium, and no butanol was added. In the thirteenth incubation, the productions of acetate, butyrate, and butanol were analyzed daily.

4.3.2 Analytical methods

The concentration of acetate, butyrate, and butanol were analyzed using an HPLC AGILENT 1260 (Agilent Technologies, CA, USA) equipped with a diode array and refractive index detectors and an AMINEX HPX-87 H column. 5 mM H_2SO_4 served as eluent at a flow rate of 0.6 mL/min with a column temperature at 50 ºC as previously reported by [9] et al., (2020).

4.3.3 Statistical analysis

Statistical significance was determined using analysis of variance using a one-way ANOVA test for comparison of means, with a significance level of 95% ($p < 0.05$). Analyses were performed using the Excel, Microsoft Office Professional Plus 2016.

4.3.4 Molecular analysis

Wet samples were collected at the end of Stages I and III to determine the bacterial composition from replicate reactors. These samples were subjected to DNA extraction using PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was quantified and sequenced using the Illumina MiSeq platform using the primer pair V3-V4 341 F (5′- CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) [15]. The analysis of the resulting sequences was performed according to [16].

4.4 Results and discussion

4.4.1 Stage I. Increasing concentrations of butanol

Stage I consisted of applying 0.25 g/L increments of butanol to microbial consortia with four initial butanol concentrations. The acetate/butyrate (A/B) ratio revealed changes in the fermentation products of the parental consortium (control) *versus* the consortia that received butanol at the beginning of each incubation (Figure 4.2A). The parental consortium always produced more acetate than butyrate, where the A/B ratio increased with the incubations with a maximum value of 9.73 in the fourth incubation. The A/B ratio in consortia that received butanol changed since the first incubation at all the initial butanol concentrations (Figure 4.2A). These consortia gradually produced more butyrate than acetate, where the consortium that received the highest butanol concentration (treatment D) reached the lowest A/B ratio of 0.26 in the sixth incubation. As the number of incubations increased, the A/B ratio between treatments was statistically different. During experimentation, the parental consortium produced a maximum butanol concentration of 1.5 g/L in the sixth incubation (Figure 4.2B). In those consortia that received butanol, it was expected that butanol would accumulate. Contrary, in most treatments/incubations, the final concentration was lower than the initial concentration leading to negative values of Δ butanol (Figure 2B). Δ butanol was greater as the number of incubations increased and as the initial butanol concentration increased. For example, in treatment A, Δ butanol increased from -0.12 in the first incubation to -1.61 in the sixth incubation, and Δ butanol increased from -1.61 for treatment A with the lowest butanol addition to -5.08 for treatment D with the highest butanol addition.

Bacterial diversity after the sixth incubation showed similar microbial consortium profiles between replicate reactors (Figure 4.2C). The parental consortium comprised 1,128 operational taxonomic units (OTUs), of which between 85 to 90 % had an abundance lower than 1%. In the parental consortium, only *Acetanaerobacterium* and Verruc-01 (belonging to the Puniceicoccaceae family) had an abundance close to 5%. The increasing addition of butanol enriched the microbial consortia with the same seven species: *Bacteroides*, *Caproiciproducens*, Incertae Sedis (belonging to the Ethanoligenenaceae family), *Anaerofilum*, *Acetanaerobacterium*, *Ruminococcus*, and Verruc-01. Some patterns could be associated with the addition of butanol. For example, some bacteria prosper at similar abundances regardless of the butanol concentration, these bacteria were *Ruminococcus* and Verruc-01. In contrast, the increasing concentrations of butanol affected the abundance of some species in at least

one replicate. *Caproiciproducens* and *Anaerofilum* prosper best in higher concentrations of butanol, but *Bacteroides* and *Acetanaerobacterium* languished.

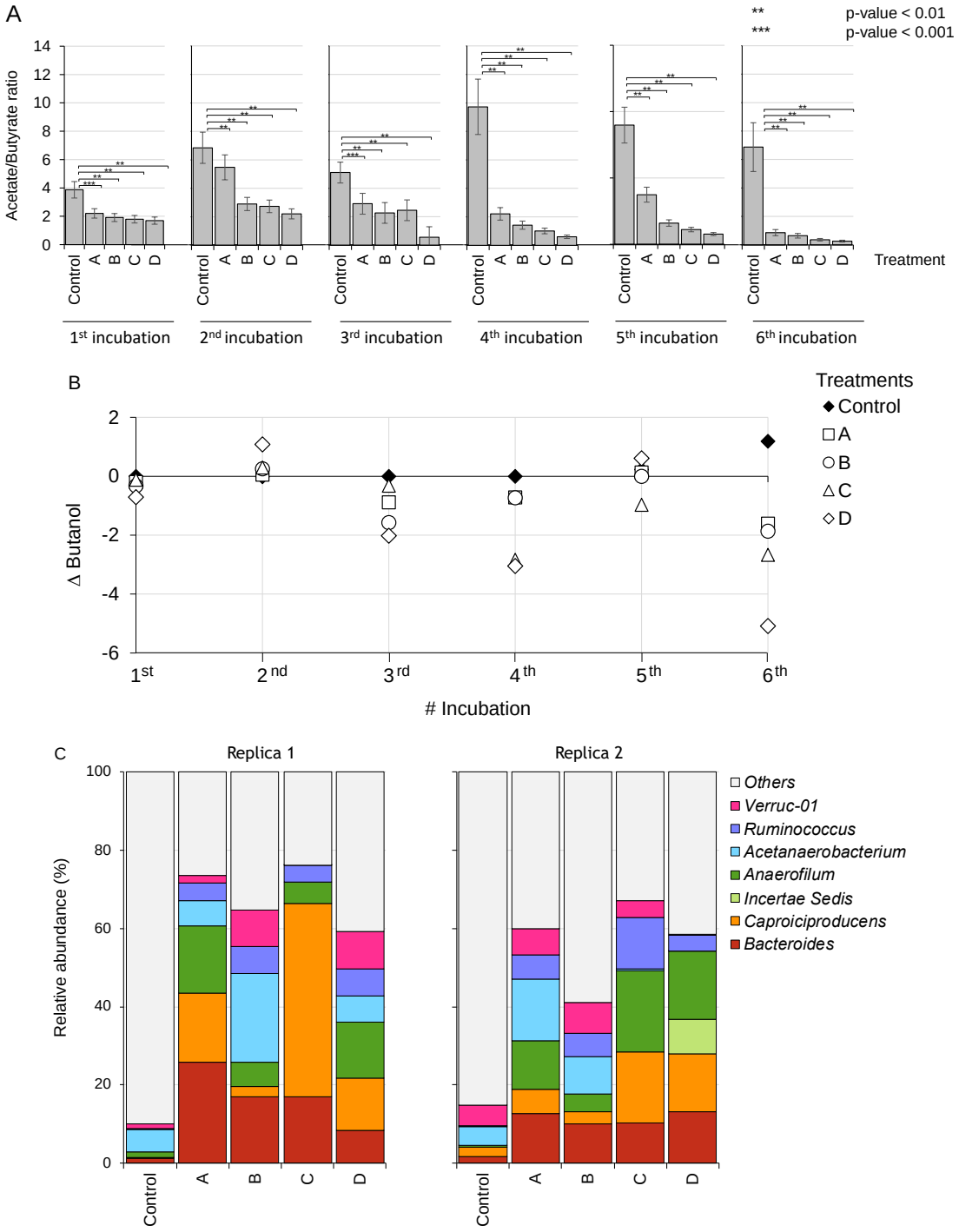

Figure 4.2. Performance of the four microbial communities during the adaptive laboratory evolution experiment (Stage I: increasing concentrations of butanol). **A.** Acetate/butyrate ratio after each incubation with 0.25 g/L increments of butanol. **B**. Δ butanol (final concentration -/minus initial concentration of butanol) after each incubation with 0.25 g/L increments of butanol. **C**. Bacteria diversity at the end of the sixth incubation obtained from duplicate reactors. Similar bacterial groups were obtained for both replicates where all major groups belonged to the phylum of Firmicutes.

4.4.2 Stage II. Absence of butanol added

During this stage, the parental consortium and the treatments were incubated without butanol added. No significant changes were observed in this stage, since the parental consortium continued producing more acetate than butyrate, opposite tendency in treatments that received butanol. At the end of each incubation, Δ butanol continued having negative values.

4.4.3 Stage III. Shock of butanol added

In this stage, all microbial consortia (except the parental consortium) received a shock of 5 g/L butanol during the eleventh and twelfth incubations. The acetate/butyrate ratio remained unchanged with values lower than 0.61 for all consortia (Figure 4.3A). At the end of the twelfth incubation, Δ butanol increased in all treatments compared to Stages I and II ($p < 0.05$). Values for Δ butanol were the lowest observed during the ALE, -9.96 for treatment A, -5.53 for treatment B, and -12.29 for treatment D (Figure 4.3B). Treatment C had similar values than to previous stages.

Bacterial diversity after the twelfth incubation showed similar species between replicate reactors (Figure 4.3C). Consortia that received butanol maintained the majority of species identified at the end of Stage 1, that is, *Bacteroides*, *Caproiciproducens*, *Anaerofilum*, *Acetanaerobacterium*, and *Ruminococcus*. But, two novel species emerged at the end of butanol shock, these species were an unknown genus belonging to the Clostridia UCG-014 Order and *Pseudomonas*. The abundance of *Pseudomonas* increased as increased the butanol added, contrary, the genus of Clostridia UCG-014 languished.

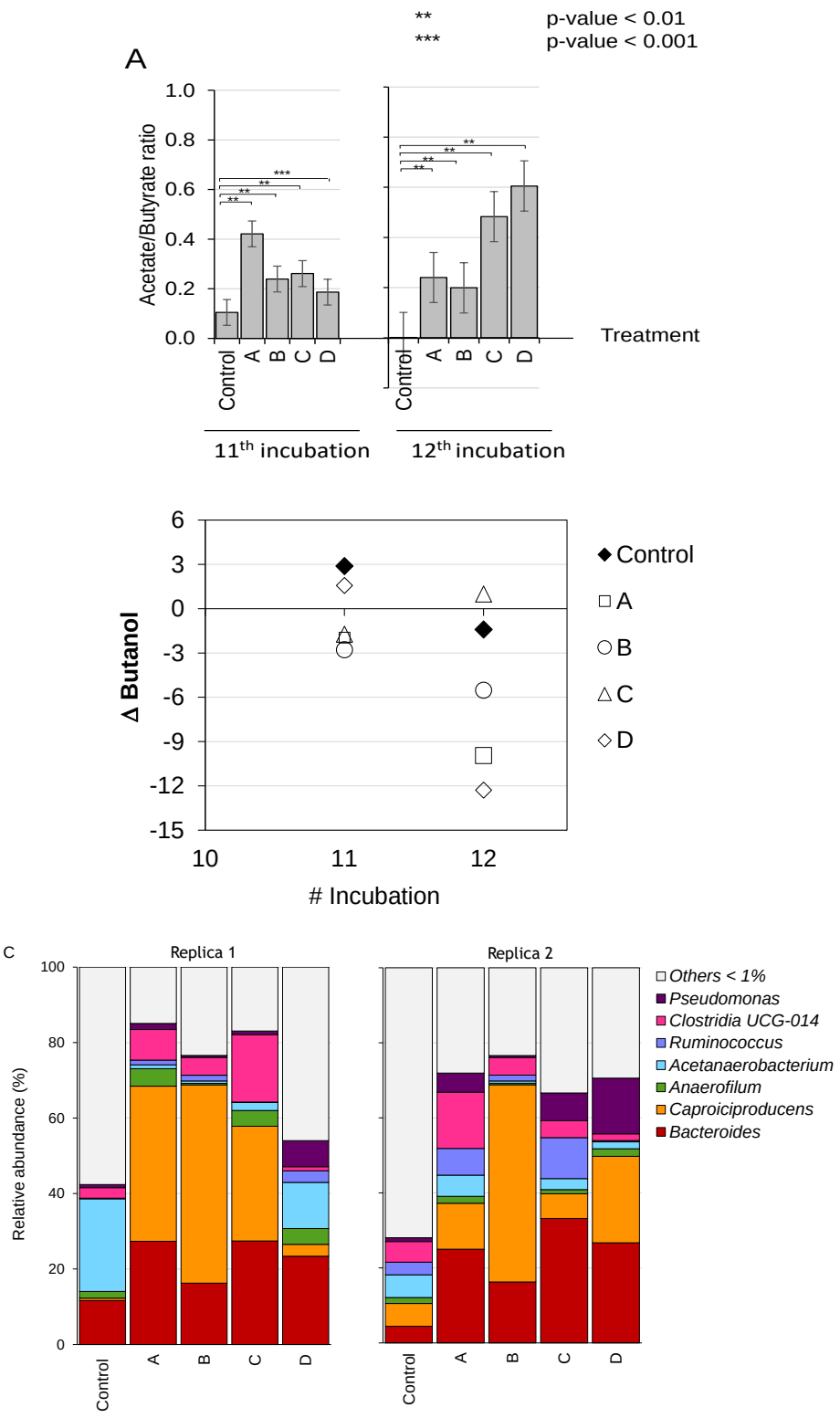

Figure 4.3. Performance of the four microbial communities during the adaptive laboratory evolution experiment (Stage III: increasing concentrations of butanol). **A.** Acetate/butyrate ratio after each incubation with 5 g/L increments of butanol. **B**. Δ butanol (final concentration minus initial concentration of butanol) after each incubation with 5 g/L increments of butanol. **C**. Bacteria diversity at the end of the two incubation obtained from duplicate reactors. Similar bacterial groups were obtained for both replicates

4.4.4 Stage IV. Kinetic of butanol production

After the butanol shock, most consortia exhibited an increased Δ butanol, suggesting that their members could be metabolizing butanol. Figure 4.4 shows the butanol production during seven days. For all consortia, the butanol concentration peaked at the fourth day between 1.1 to 13.8 g/L, after, the butanol concentration decreased. The butanol concentration increased as the butanol added increased during Stage I, thus, treatment D yield the highest butanol concentration. These results confirm that butanol is metabolized by some members in the consortia after four days of incubation.

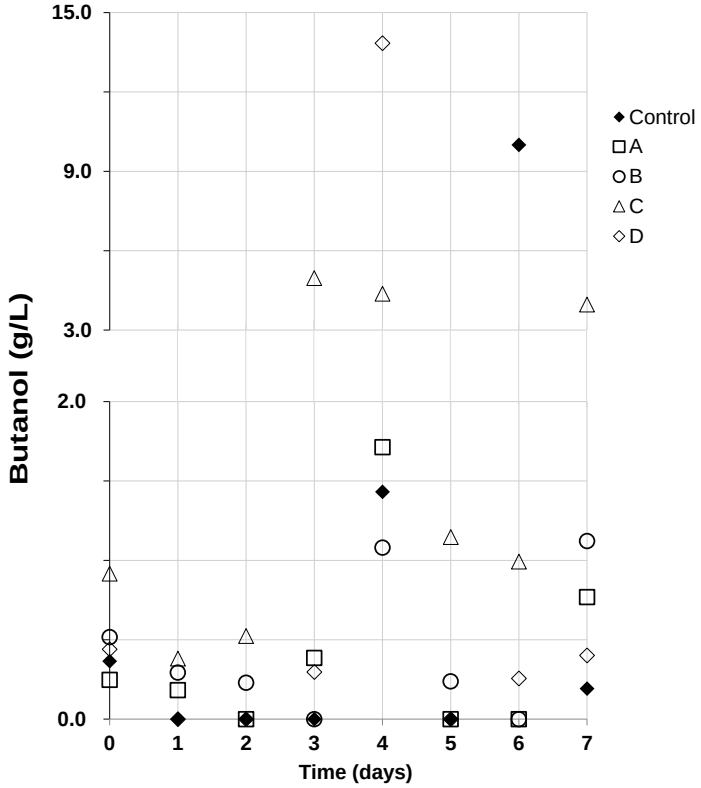

Figure 4.4. Butanol production during seven days in Stage IV.

4.5 Conclusions

Adaptive laboratory evolution was successfully applied to an acidogenic consortium for enhancing their butanol tolerance. After six incubations in presence of butanol, the parental consortium changed their microbial structure and fermentation products. The resulting consortia were enriched with *Bacteroides*, *Caproiciproducens*, *Anaerofilum*, *Acetanaerobacterium*, and *Ruminococcus*, species possibly involved in the substrate hydrolysis and production of short-

chain fatty acids. Also, an unknown genus belonging to the Clostridia UCG-014 Order and *Pseudomonas* were identified when the butanol added increased. *Pseudomonas* could be responsible of biocatalysis of butanol while the unknown genus of Clostridia UCG-014 could be responsible of butanol production.

4. 5 References

[1] Pugazhendhi A, Mathimani T, Varjani S, Rene ER, Kumar G, Kim SH, Ponnusamy VK, Yoon JJ. Biobutanol as a promising liquid fuel for the future - recent updates and perspectives. Fuel 2019; 253:637-646. https://doi.org/10.1016/j.fuel.2019.04.139.

[2] Xue C, Cheng C. Chapter Two - Butanol production by Clostridium. In Editor(s): Li Y, Ge X. Advances in Bioenergy. Elsevier., 2019;4: 35-77. https://doi.org/10.1016/bs.aibe.2018.12.001.

[3] Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. Fermentative butanol production by Clostridia. Biotechnol. Bioeng 2008;101(2):209–228. https://doi.org/10.1002/bit.22003.

[4] Mavrommati M, Daskalaki A, Papanikolaou S, Aggelis G. Adaptive laboratory evolution principles and applications in industrial biotechnology. Biotechnol. Adv. 2021;107795. https://doi.org/10.1016/j.biotechadv.2021.107795.

[5] Xu M, Zhao J, Yu L, Yang S-T. Comparative genomic analysis of Clostridium acetobutylicum for understanding the mutations contributing to enhanced butanol tolerance and production. J. Biotechnol. 2017; 263: 36-44. https://doi.org/10.1016/j.jbiotec.2017.10.010.

[6] Dragosits M, Mattanovich D. Adaptive laboratory evolution – principles and applications for biotechnology. Microb. Cell Fact. 2013;12:64. https://doi.org/10.1186/1475-2859-12-64.

[7] Liu R, Chen Y, Tian Z, Mao Z, Cheng H, Zhou H, Wang W. Enhancing microbial community performance on acid resistance by modified adaptive laboratory evolution. Bioresour. Technol. 2013;287:121416. https://doi.org/10.1016/j.biortech.2019.121416.

[8] Wen Z, Ledesma-Amaro R, Lin J, Jiang Y, Yang S. Improved n-Butanol Production from Clostridium cellulovorans by Integrated Metabolic and Evolutionary Engineering. Appl. Environ. Microbiol. 2019;85(7): e02560-18. https://doi.org/10.1128/AEM.02560-18.

[9] González-Tenorio D, Muñoz-Páez KM, Valdez-Vazquez I. Butanol production coupled with acidogenesis and CO_2 conversion for improved carbon utilization. Biomass Conv. Bioref 2020. https://doi.org/10.1007/s13399-020-00805-y.

[10] Valdez-Vazquez I, Poggi-Varaldo MH. Hydrogen production by fermentative consortia. Renew Sustain Energy Rev 2009;13(5):1000-1013. https://doi.org/10.1016/j.rser.2008.03.003.

[11] Rodríguez F, Sanchez A, Parra C. Role of Steam Explosion on Enzymatic Digestibility, Xylan Extraction, and Lignin Release of Lignocellulosic Biomass. ACS Sustain. Chem. Eng. 2017;5(6):5234–5240. https://doi:10.1021/acssuschemeng.7b00580.

[12] Ding J, Luo H, Xie F, Wang H, Xu M, Shi Z. Electron receptor addition enhances butanol synthesis in ABE fermentation by Clostridium acetobutylicum. Bioresour. Technol. 2018; 247:1201-1205. https://doi.org/10.1016/j.biortech.2017.09.010.

[13] Vasylkivska M., Patakova P., Role of efflux in enhancing butanol tolerance of bacteria, J. Biotechnol., 2020; 320: 17-27. https://doi.org/10.1016/j.jbiotec.2020.06.008.

[14] Reyes L. H., Almario M. P., Winkler J., Orozco M. M., Kao K. C., Visualizing evolution in real time to determine the molecular mechanisms of n-butanol tolerance in *Escherichia coli*, Metab. Eng., 2012; 14 (5): 579-590, https://doi.org/10.1016/j.ymben.2012.05.002.

[15] Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41(1):e1. https://doi.org/10.1093/nar/gks808 e1.

[16] Carrillo-Reyes J, Buitrón G, Arcila JS, López-Gómez MO. Thermophilic biogas production from microalgae-bacteria aggregates: biogas yield, community variation and energy balance. Chemosphere (Oxford) 2021. https://doi.org/10.1016/j.chemosphere.2021.129898, 129898.

Capítulo 5.0 Resultados. Fermentación de los residuos sólidos orgánicos y de los residuos gaseosos de la acidogénesis para la obtención de productos de valor agregado

El capítulo 5 estuvo dedicado al cumplimiento del objetivo específico 4, la valorización de los residuos orgánicos y gaseosos CO_2/H_2 (off gas) generados en la acidogénesis para la producción de ácidos de cadena corta (AGCC) y etanol, como precursores de ácidos grasos de cadena media (AGCM) como el caproato. Para ello, se trabajó con la fermentación de gases utilizando consorcios autotróficos. De los resultados del objetivo sobre la fermentación acidogénica (Capítulo 3) se consideró una mezcla sintética de H_2/CO_2 y los AGV (acetato y butirato) para la producción de AGCM.

La motivación del trabajo fue demostrar que los subproductos de la acidogénesis, AGCC y gases, pueden ser utilizados como sustrato para incrementar el rendimiento de productos de una biorrefinería.

La estrategia fue evaluar el efecto del tipo de inóculo sobre la producción de AGCM. Los inóculos empleados fueron: 1) digestatos y 2) lixiviados de una fermentación acidogénica, y 3) lodo anaerobio granular. Los reactores tuvieron un volumen efectivo de 100 mL y fueron operados en modo semicontinuo durante 147 días. En la primera etapa, se alimentó el reactor con una mezcla de H_2/CO_2 hasta observar el consumo de H_2 > 90%. Después, la segunda etapa consistió en adicionar H_2/CO_2 sin remplazar el sobrenadante donde se han acumulado los productos de fermentación. Durante la primera etapa, los reactores con digestatos produjeron AGCC en concentraciones entre 4,274 y 5,381 mg/L, los reactores con lodos produjeron 3,528 mg/L de solventes (principalmente etanol), y los reactores con lixiviados produjeron 617 mg/L de caproato. Durante la segunda etapa, cuando se tuvo una atmósfera enriquecida en CO_2, los reactores con lixiviados produjeron 6,157 mg/L de caproato a partir de la condensación de los AGCC y los solventes, presumiblemente a través de la vía de oxidación β inversa. En los reactores con digestatos y lixiviados se identificaron miembros autotróficos

como *Proteiniphilum* y *Acinetobacter,* especies de bacterias que llevan a cabo la elongación de los AGCC por la vía de oxidación β inversa. En los reactores con lodos, se identificaron bacterias de los géneros *Clostridium* y *Desulfovibrio*, posibles responsables de la oxidación β inversa.

El estudio del proceso de fermentación de gases permitió considerar la producción del ácido caproico, como otra posibilidad para el aprovechamiento del sustrato en biorrefinerías lignocelulósicas. Para ello, se tendría que proponer un nuevo modelo de biorrefinería que integrara la biofijación de CO_2, estudiada en el objetivo específico 5 (capítulo 6). Sin embargo, aún hacen falta más estudios sobre este tema, por ejemplo, estudiar otras composiciones de gases semejantes a las obtenidas en la acidogénesis. Otro factor muy importante, es estudiar el proceso de fermentación de gases en reactores en continuo que permitan trabajar con tiempos de retención cortos, similar a estudios reportados en la literatura que han utilizado otras composiciones de gases.

Referencia del trabajo publicado

González-Tenorio D, Muñoz-Páez KM, Buitrón G, Valdez-Vázquez I. 2020. Fermentation of organic wastes and CO_2 + H_2 off-gas by microbiotas provides short-chain fatty acids and ethanol for n-caproate production. J CO_2 utilization 42,101314. https://doi.org/10.1016/j.jcou.2020.101314.

5.1 Abstract

This study describes a two-stage process for the valorization of organic wastes and CO_2/H_2 off-gas for the production of short-chain fatty acids (SCFAs) and ethanol, as precursors for *n*-caproate formation. Three complex organic wastes with previously identified acetogenic members were used to inoculate batch reactors: 1) fibers and 2) leachates from a bagasse fermentation process, and 3) an anaerobic granular sludge from a reactor treating brewery effluents. In the first stage, the reactors loaded with a CO_2/H_2 [25:75, v/v] gas mixture produced SCFAs at similar concentrations between 4,274 and 5,381 mg/L. In this stage, fiber reactors had the highest H_2 consumption. In the second stage, reactors consumed all available H_2 and accumulated solvents as well as low concentrations of *n*-caproate. In particular, sludge reactors produced 3,528 mg/L of solvents with more than 50% ethanol content, and leachate reactors produced 617 mg/L of *n*-caproate. By the end of the second stage, in a CO_2-enriched atmosphere, leachate reactors produced 6,157 mg/L of *n*-caproate at the expenses of the SCFAs and solvents. Fiber and leachate microbiotas shared autotrophic members and SCFA elongating bacteria while sludge microbiota differed in composition. This study demonstrated that the use of CO_2/H_2 off-gas shifted the composition of SCFAs to a mixture with ethanol which promoted its condensation into *n*-caproate. A CO_2-enriched atmosphere improved significantly the final production of *n*-caproate. The results of this study can be used to integrate the *n*-caproate production, and possibly other medium-chain fatty acids, with biorefinery schemes in which SCFAs and CO_2/H_2 off-gases are produced

Keywords: acetogens; biogenic CO_2; biorefineries; hydrogen consumption; reverse β oxidation

5.2 Introduction

A biorefinery is defined as a plant that converts biomasses to energy, biofuels, chemicals, and biomaterials [1]. While considered environmentally friendly, biorefineries generate large amounts of CO_2 undermining their sustainability [2]. In general, biorefineries have three main sources of CO_2 emissions. First, the combustion of solid lignin-rich residues for the generation of heat, electricity, and steam emits flue gases containing CO_2 (< 20%) together with NOx, SOx, CO, VOCs. Second, the anaerobic digestion process to produce CH_4 also generates CO_2 (< 40%) together with N_2, O_2, H_2S, NH_3. Third, microbial respiration oxidizes substrates, producing high-purity CO_2 (≥ 95-96%) in ethanol plants and CO_2/H_2 off-gases (H_2 between 30 to 40%) in

acidogenesis and acetone-butanol-ethanol (ABE) fermentation processes [3,4,5]. Biological sequestration of CO_2, the process of capturing the CO_2 generated by anthropogenic activities to convert it to biofuels and chemicals, can be used as a measure to reduce greenhouse gas emissions in biorefineries [6]. Autotrophs are capable of performing CO_2 bio-sequestration, of particular interest are autotrophic acetogens that use the Wood-Ljungdahl pathway to couple H_2 oxidation to CO_2 reduction for the generating of short-chain fatty acids (SCFAs) as valuable fermentation products [7]. The CO_2/H_2 off-gases seem to be one excellent CO_2 sources for SCFA production because of the concurrent presence of H_2 [4]. So far, researchers have used CO_2/H_2-containing off-gasses in two ways. One way is to recirculate the off-gas to improve the overall product yield. For example, enriching reactor headspace with CO_2/H_2 off-gasses (approx. 1:1) increased 1.2 times the production of succinic acid from glucose fermentation [4], and 1.4 and 2.5 times the production of SCFAs from corn stover and organic waste fermentation, respectively [8,9]. The second approach uses CO_2/H_2 off-gases as the sole carbon source in gas fermentation. In this case, gas fermentation operational parameters affecting CO_2 fixing can be controlled to direct fermentation towards one target product. For example, previous studies using CO_2/H_2 as the sole substrate obtained between 1,300 to 4,000 mg/L of fermentation products, and reported the selective reduction of CO_2 to formic acid (83%) using a pure culture of *Clostridium ljungdahlii* [10], and to acetic acid (64%) and butanol (73%) by using selectively enriched microbiotas [5,11]. However, the major limitation of this approach is the low SCFA concentration and, consequently, high costs for product recovery and purification [12]. To overcome this limitation, SCFAs such as acetate, propionate, and *n*-butyrate can be condensed to medium-chain fatty acids (MCFAs) such as *n*-caproate, *n*-heptanoate, and *n*-caprylate, *via* the reverse β-oxidation pathway [13]. The MCFAs are saturated fatty acids that have chains with 6 to 12 carbons, with high economic value and are used in pharmaceuticals and fuel production [14]. Many gaseous substrates can be used to produce SCFAs as intermediate metabolites for MCFAs synthesis, for example, CO_2 from biorefineries, CO_2 from industrial waste, CO from syngas, and CO_2/CH_4 from anaerobic digestion [3, 6]. So far, only CO-rich syngas fermentation effluents were reported to produce *n*-caproate at concentrations up of 1,000 mg/L [12]. This study aims at testing three organic wastes and its respective microbiotas to produce SCFAs and ethanol from CO_2/H_2, and then to condensate them into MCFAs. The wastes (solid and liquid) and its microbiotas were obtained from acidogenic digestion of bagasse containing metabolically active autotrophic acetogens

[15], and sewage sludge from an up flow anaerobic sludge bed (UASB) reactor treating brewery effluents containing clostridial acetogens [16]. These three organic wastes include typical residual streams produced as a by-product in advanced refineries. For example, in biorefineries where acidogenesis is the previous stage of methanogenesis, part of the produced SCFAs (or spent solids) can be derived to produce methane or to condensate them into MCFAs. In ABE fermentation processes, the rejected streams after the purification stage (with SCFAs and ethanol) can be also derived to a condensation process. Finally, sewage sludge produced during the wastewater treatment in advanced refineries can also serve as substrate or source of microorganisms to produce SCFAs, solvents or MCFAs [2, 4, 5]. The intention of this research was elucidated if each one of these substrates could be used interchangeably to produce SCFAs and MCFAs despite its different composition.

5.3. Materials and methods

5.3.1. Organic wastes and inocula

Organic wastes with its respective microbiotas used for off-gas fermentation were fibers and leachates from a fermenter processing agave bagasse whose compositions were: fibers, extractives 28.3 %, hemicellulose 21.9 %, cellulose 42.4 %, and lignin 7.4 %; and leachates, soluble chemical oxygen demand 30 g/L, total carbohydrates 4.7 g/L, acetic acid 3.7 g/L, butyric acid 0.4 g/L, and propionic acid 0.5 g/L [15], and an anaerobic granular sludge from a full scale UASB reactor treating brewery effluents with a volatile solid content in the dried sludge of 0.4 g/g [17]. The three organic wastes were thermally treated to eliminate methanogens which could compete for the substrate to produce CH_4 [11]. The medium culture used consisted of 4 g/L of urea and 2 g/L of KH_2PO_4. The gaseous substrate was a synthetic mixture of high purity CO_2 (≥99.0%) and H2 (≥99.8%), 25:75 (v/v) according to Modestra et al. [11].

5.2.2. Reactors set-up and experimental design

The off-gas fermentation reactors consisted of 250 mL glass serum bottles with a working volume of 100 mL. A single factor experiment at three levels was used to study the effects of waste/inoculum type on the productions of SCFAs, solvents and MCFAs from the H_2+CO_2 fermentation. The three organic wastes with its respective microbiotas were: 5 g of dry fibers (fiber reactors), 5 mL of non-sterile leachates (leachate reactors), or 5 g of dry anaerobic sludge (sludge reactors). The reactors were flushed with H_2/CO_2 and set at 1.28 bar before incubation at 37 ◦C and 150 rpm on an orbital shaker in duplicate, the pH was adjusted to 7.0. The H_2 consumption was taken as the reference point to define each operation stage. Once each

reactor consumed 90% of H_2, the second stage started with fresh H_2/CO_2 mixture and without replacement of supernatant to promote metabolite conversions in subsequent operation stages (Fig. 5.1).

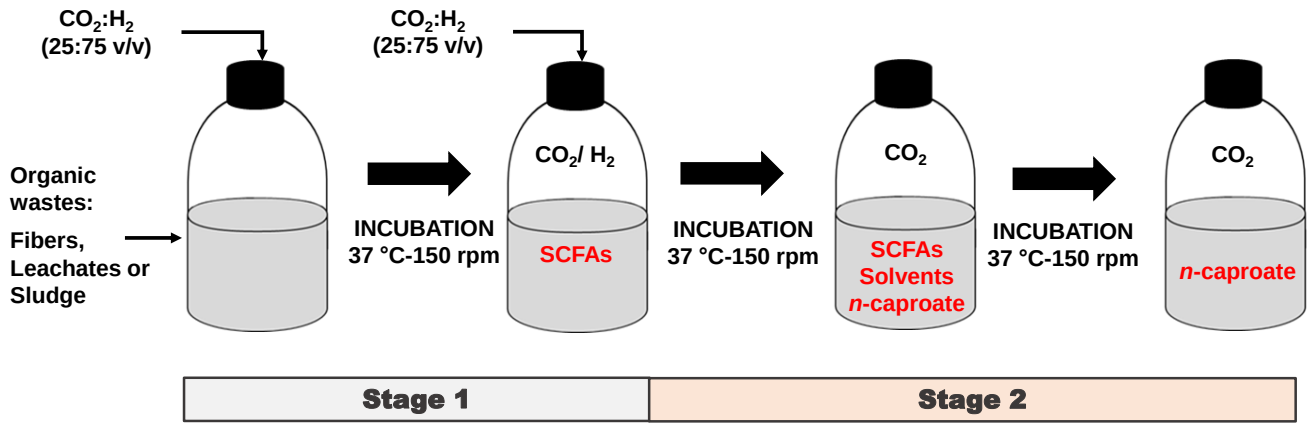

Fig. 5.1. Flow chart depicting the experimental methodology

5.3.3. Analytical methods

The composition of the biogas was measured weekly using an SRI 8610C gas chromatograph (IL, USA) equipped with a thermal conductivity detector and two packed columns as was previously reported by Munoz-Páez et al. [17]. The concentrations of fermentation products were determined once each reactor consumed all available H_2 or when its concentration remained unchanged for several days (*i.e.*, at Days 12, 147, and 167). The SCFA concentrations (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, and caproic acids) y solvents (acetone and ethanol) were determined using a Varian 3300 gas chromatograph (Palo alto, CA, USA) connected to a flame ionization detector and a 15-m long (0.53 mm id) Zebron ZB-FFAP column according to the procedure reported previously [5]. The concentration of butanol was determined using an Agilent 1260 high-performance liquid chromatograph (Agilent Technologies, CA, USA) equipped with a diode array and refractive index detectors, and an Aminex HPX-87H column, using the conditions reported previously [5].

5.3.4. Molecular and statistical analysis

Bacterial diversity of each reactor was determined at the initial day after the pretreatment (Day zero) and at the end of the experiment by sequencing amplicons of 16S rRNA gene (V1-V3

variable region) using the Ilumina MiSeq platform. Genomic DNA was extracted from selected samples with the PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration and quality were determined by spectrophotometry using a NANODrop 2000c (Thermo Scientific, Wilmington, DE, USA). The sequences obtained was analyzed according to Munoz-Páez et al. [16]. The abundance of major taxa (>3%) were used to generate Jaccard distance matrices for the bootstrapped datasets, and to generate an input file for a consensus neighbor-joining tree. A canonical correspondence analyses (CCA) were performed to model the operational taxonomic units (OTUs) to production of *n*-caproate and solvents. Both analyzes were performed using the PAST software v4.03 [18]. Design-Expert v10 (Stat-Ease, MN, USA) was also used for a one-way ANOVA to test statistical difference between samples taken from different reactors and times.

5.4 Results and Discussion

This study reports the effects of three complex organic wastes with acetogenic members on production of SCFAs, solvents, and MCFAs. Data reported correspond to an acclimation phase in which the H_2 consumption was taken as the reference point to define each operation stage, and thus, to obtain data about concentration of fermentation end products. During the first 12 days of incubation, the three microbiotas consumed different amounts of H_2 (p-value < 0.001). Microorganisms in the fiber reactors had the highest H_2 consumption while the leachate and sludge reactors consumed H_2 equally (Fig. 5.1A). The total H_2 consumption in the fiber reactors enriched the atmosphere in CO_2 (>99%) while the CO_2 concentration in the leachate and sludge reactors was 42 ± 6% due to presence of unconsumed H_2 (Fig. 5.1B). Methane production was negligible in the first stage (Fig. 5.1C). During the first stage, H_2 consumption in fiber reactors was higher in comparison with previous studies that used an anaerobic inoculum receiving a similar mixture of CO_2/H_2 [20:80 v/v] with 50% H_2 consumption after 30 days of incubation [11]. It is likely that bagasse polysaccharides present in the fiber reactors promote H_2 consumption [8]. In the second stage, all tested microbiotas consumed all available H_2. By Day 84, the fiber and leachate reactors had both the faster H_2 consumption in comparison with the sludge reactors (p-value < 0.01, Fig. 5.1A). By Day 147, the atmosphere in all reactors was enriched in CO_2 (p-value > 0.05, Fig. 5.1B). At this stage, all reactors accumulated similar amounts of CH_4 (p-value > 0.05, Fig. 5.1C). These results were similar to those reported for an untreated anaerobic inoculum accumulating methane up to 19% after 16 days of incubation [11]. CH_4

accumulation in the second stage is likely due to the activity of methanogens that compete with acetogens for H_2. At the start of the experiment (Day zero), all reactors contained small amounts of SCFAs between 855 mg/L (leachate reactors) and 1,076 mg/L (fiber reactors) as a consequence of the fermentation processes from which each organic waste came. MCFA and solvent concentrations were below 53 mg/L and 61 mg/L, respectively. By Day 12, all reactors produced SCFAs at similar concentrations between 4,274 ± 910 and 5,381 ± 148 mg/L (p-value > 0.05, Fig. 5.1D), acetic acid being the most abundant fermentation product (48-56%, Table 5.1).

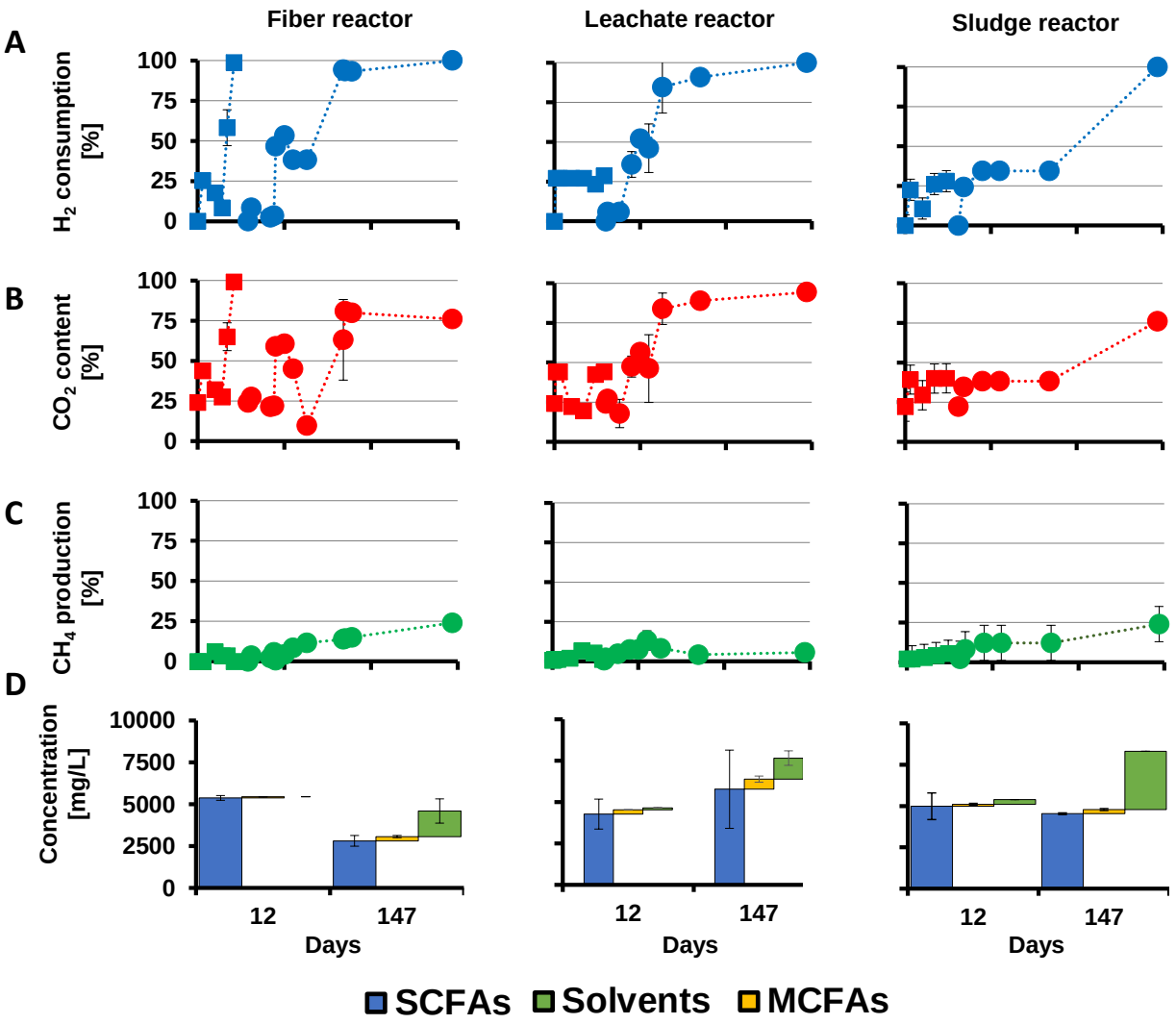

Fig. 5.1. First (squares) and second (circles) stage of organic waste and CO_2/H_2 off-gas fermentation using microbiotas from different sources. SCFAs, short-chain fatty acids including acetate, butyrate, propionate, iso-butyrate, valerate, iso-valerate; MCFAs including *n*-caproate; Solvents including ethanol, acetone and butanol.

Previous studies that combined CO_2/H_2 off-gas fermentation of with sugar fermentation to produce SCFAs, achieved concentrations greater 18,000 mg/L [4, 9]. In contrast, fermentation of H_2/CO_2 by an acid- and heat-treated inoculum achieved a ten time lower SCFA concentration after 15 days of incubation [11]. In this study, fiber reactor reached the highest SCFA concentrations (5,381 ± 148 mg/L) after a comparable incubation time (12 days). For the second stage, reactors were flushed with fresh H_2/CO_2 mixture without replacement of culture medium

that contained the cumulated fermentation products. By Day 147, fiber reactors accumulated lower SCFA concentrations in comparison with the first stage (p-value < 0.05) whereas that the leachate and sludge reactors accumulated similar SCFA concentrations (p-value > 0.05, Fig. 5.1D). Regarding the production of solvents and MCFAs, all reactors produced higher concentrations in comparison with the first stage (all p-values < 0.05, Fig. 5.1D).

Solvents represented between 16% to 42% of total end products; the sludge reactor produced the highest amount of solvents (3,528 mg/L), 52% of which being ethanol (Table 5.1). Ethanol is produced *via* the Wood–Ljungdahl pathway, a set of biochemical reactions used by acetogens to reduce CO_2 (electron acceptor) with H2 (electron donor) producing acetate and subsequently ethanol [19]. On the other hand, MCFAs –represented by *n*-caproate– increased their percentage from 3% in the first stage to 9% in the second stage; leachate reactors produced the highest of *n*-caproate (617 ± 186 mg/L) (Fig. 5.1D). Previous studies addressing the effects of H_2/CO_2 headspaces on corn stover and organic waste fermentation reported similar concentrations of *n*-caproate: from a minimum of 118 mg/L after 28 days of incubation with a heat-treated anaerobic granular sludge, to a maximum of 1, 600 mg/L after 35 days of incubation with adapted marine microorganisms [8,9]. After the H_2-exhaustion phase and the resulting enrichment in CO_2, reactor incubation time was prolonged 20 days to observe changes in the composition of fermentation products [12]. Leachate reactors achieved a maximum production of *n*-caproate (6,157 mg/L) at the expenses of SCFAs and solvents. In the other reactors, SCFA and solvent concentrations decreased below 150 mg/L, but *n*-caproate could not be identified as the main final fermentation product as previously has been reported [8,9], possibly due to the selective production of other MCFAs such as *n*-heptanoate, and *n*-caprylate, fact that needs further confirmation. CO_2-enriched atmospheres favor the elongation of SCFAs, resulting in *n*-caproate production *via* the reverse β-oxidation pathway [12]. In the reverse β-oxidation pathway, SCFAs such as acetate, propionate, and butyrate act as electron acceptors while ethanol acts as the electron donor [13]. Here we show that SCFAs produced in the first stage through organic waste and gas fermentation, and ethanol produced during the second stage *via* the Wood–Ljungdahl pathway, together with the CO_2-enriched atmosphere, favored the *n*-caproate production *via* the reverse β-oxidation pathway. We obtained a five-time higher *n*-caproate concentration with respect to previously reported amounts (1,000 mg/L) using a mixed culture and SCFAs from CO-rich syngas fermentation [12], and a similar concentration to that obtained in a continuous anaerobic upflow reactor fed with synthetic mixtures of acetic

acid and ethanol (7,500 mg/L) [13]. However, we observed *n*-caproate accumulation at a later time (167 days), compared to what observed in other studies (24 and 98 days). These differences may be due to a lack of previous acclimation of the microbiotas to the elongation of SCFAs. Future studies should explore more deeply the metabolic shifts during operation stages to reduce the times required to produce each type of fermentation product, *i.e.*, SCFA, solvents, and MCFAs.

Table 5.1. Composition of fermentation-end products at the end of first stage (day 12) and second stage (day 147).

Reactor	Day	Short-chain fatty acids (%)						Solvents (%)		
		Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Valeric acid	Isovaleric acid	Acetone	Ethanol	Butanol
Leachate	**12**	55.5	11.2	2.7	27.7	2.5	0.4	0.0	100	0.0
	147	61.1	14.5	1.4	14.8	6.0	2.2	43.9	54.2	1.9
Fiber	**12**	51.3	16.0	1.9	29.9	0.9	0.0	0.0	0.0	0.0
	147	47.8	12.9	7.3	19.0	8.8	4.2	69.8	20.5	9.7
Sludge	**12**	54.9	9.3	4.9	22.1	1.2	7.6	0.0	83.4	16.6
	147	18.6	5.0	2.1	12.4	14.4	47.5	23.5	52.0	24.5

In the first day of incubation, bacterial composition differed notably between inocula. Fiber and leachate microbiotas, both from the same fermentation process of agave bagasse consisted mainly of 61% *Lactobacillus*, 18% *Clostridium*, 2% *Enterococcus*, the rest of other bacteria and without presence of archaea. In contrast, sludge inoculum had an even bacterial distribution consisting of 14.5% Thermotogales, 6.3% *Thermoanaerobacter*, 5.1% *Candidatus Cloacimonas*, 3.9% Sulfurimonas, 2.3% *Sulfuricurvum*, 2.1% Syntrophobacterales, between 1.0% and 2.0% *Chryseobacterium, Arcobacter, Shewanella, Pseudomonas, Spirochaetales, Synergistales, Deltaproteobacteria*, and the rest of other bacteria, with the presence archaea, 96.5% *Methanolinea*. After the whole period of incubation, twenty-nine operational taxonomic units (OTUs) represented the major taxa across the tested microbiotas (Fig. 5.2A).

Both fiber and leachate microbiotas shared OTUs such as *Janthinobacterium, Stenotrophomonas, Proteiniphilum* and *Dechloromonas*, all reported as autotrophs [20, 21]. From these genera, possible responsible for H_2 consumption were: *Janthinobacterium* who presents a capnophilic metabolism consuming CO_2 and H_2 in conjunction with SCFA production [22, 23], and *Dechloromonas* a hydrogen-oxidizing bacterium [24]. Other common taxa in these microbiotas were an OTU belonging to *Proteobacteria, Pseudomonas, Rhodobacter, Acinetobacter and Bacteroides*. The sludge microbiota differed in composition, with a prevalence of *Enterobacter, Pseudomonas, Symbiobacterium,* and *Spirochaetales*, with some members expressing CO_2-fixing enzymes [25, 26]. Due to their common bacterial structure, fiber and leachate microbiotas clustered together whereas the sludge microbiota segregated (Fig. 12 B). Besides autotrophs, the first cluster –fiber and leachate microbiota–contained microorganisms such as *Proteiniphilum* and *Acinetobacter* previously described as involved in the reverse β-oxidation pathway [14, 27]. Differently, in the sludge microbiota cluster, bacteria of the *Clostridium* and *Desulfovibrio* genera were likely responsible for the reverse β-oxidation reactions [14, 28]. Canonical correspondence analysis was performed to find relationships between species with gradients in concentration of solvents and MCFAs and to represent these relationships in an ordination space. CCA positively correlated the abundance of six taxa in the leachate reactors with the production of *n*-caproate (Figure 5.2C).

Among these taxa, the *Stenotrophomonas* genus is known to carry out CO_2 fixation *via* the Calvin-Benson-Bassham pathway [21]. Also, members of the Proteobacteria phylum were abundant in microbial communities producing SCFAs and *n*-caproate from switchgrass supplemented with ethanol [29], or butyrate, precursor of *n*-caproate [30]. Thus, microbiotas in leachate reactors seem to perform several tasks to produce, from complex carbohydrates and CO_2/H_2, SCFAs, ethanol, and finally *n*-caproate. On the other hand, the abundance of seven members of the sludge community was positively correlated with ethanol production (Figure 5.2C). In this context, one member of *Enterobacter* –one the most abundant taxa in the sludge microbiota– was used to produce ethanol from crude glycerol and tuna condensate [31], and other *Enterobacter* species was detected during the simultaneous production of ethanol and hydrogen from sweet potato [32]. Furthermore, *Spirochaetales* and *Desulfovibrio*, present in the sludge microbiota, have members with the capacity to produce ethanol from carbohydrates [33]. Notably, some *Clostridium* genera produce ethanol from the carbohydrate fermentation [34], and, from the syngas fermentation [35, 36].

This study demonstrated that fermentation of three complex organic wastes enriched with CO_2/H_2 off-gas produced comparable amounts of SCFAs (first stage), indicating functional redundancy of microbiotas despite differences in substrate and bacterial composition. This phenomenon has been previously reported during the SCFA production from different carbohydrates [37]. After the SCFA accumulation, microbiotas used available H_2 to produce ethanol, possibly from the previously accumulated acetate using the Wood–Ljungdahl pathway (second stage). Also, part of the H_2 consumed was utilized to produce CH_4. Concomitant production of SCFAs and ethanol promoted the condensation process to *n*-caproate, 617 mg/L, but not beyond. Two environmental conditions determined the improvement in the *n*-caproate production up to 6,000 mg/L, a CO_2-enriched atmosphere, and additional incubation time. The ethanol and *n*-caproate productions were carried by different specialized microbiota.

The current biorefinery proposals are an alternative option to convert different types of biomasses into energy replacing petroleum derivatives. However, these facilities are sources of CO_2 emissions, which is an important greenhouse gas contributing to climate change. This research presents an option to capture and convert CO_2/H_2 off-gases avoiding its release into the atmosphere. CO_2/H_2 off-gases can be used to produce mixtures of SCFAs with ethanol, and then, CO_2-enriched streams to condensate these fermentation products into MCFAs, which represent blocks of drugs, biopolymers or advanced biofuels with significant economic value.

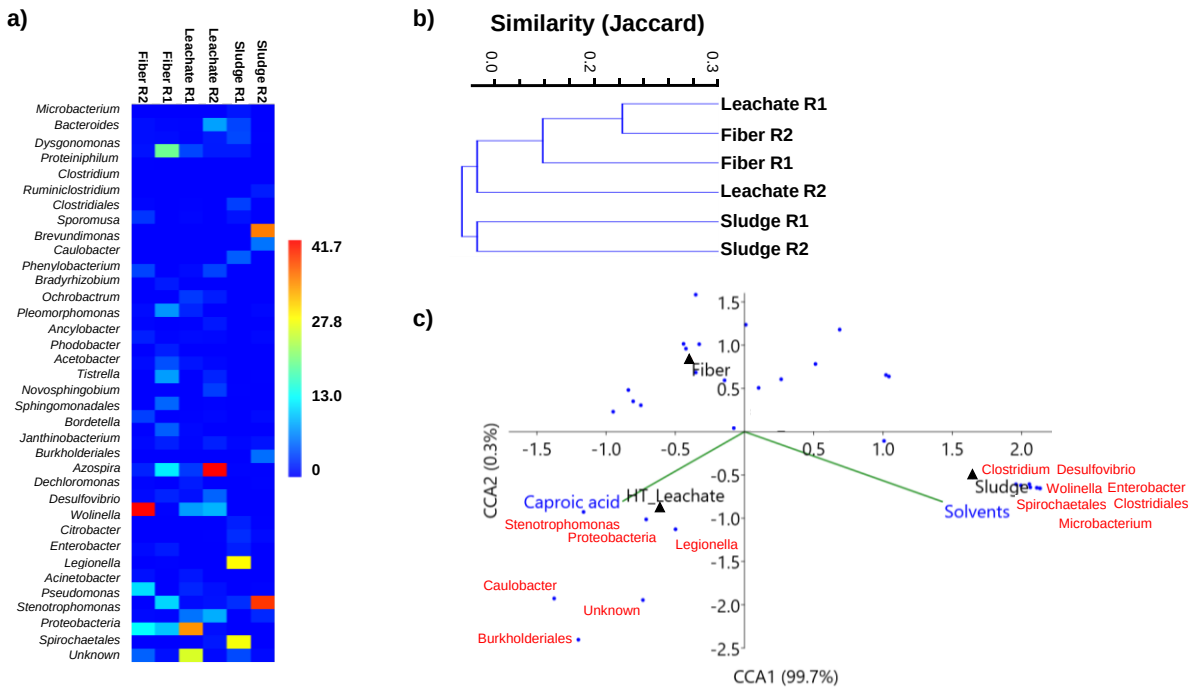

Fig. 5.2 a) Relative abundances of operational taxonomic units (OTUs) across microbiotas during the phase of *n*-caproate production. Each of these OTUs listed comprised at least 3% of the relative abundance for one or more of the samples collected. b) Neighbor-joining tree constructed from the Jaccard distance matrix of major abundant genera across microbiotas. (c) Canonical correspondence analysis (CCA) triplot showing the relationships among samples (tringles), species (circles) and metabolites (arrows).

5.4 Conclusions

This study demonstrated that the selected organic wastes spite of having different composition included autotrophic microbiotas able to produce similar amounts of SCFAs over a 12-day time span. The use of CO_2/H_2 off-gas during the fermentation promoted the accumulation of ethanol that in conjunction with acetate, previously produced from complex substrates, were condensed into *n*-caproate. A CO_2-enriched atmosphere improved significantly the production of *n*-caproate. The ability to produce SCFAs was shared between all microbiotas, but the ability to produce ethanol or *n*-caproate was specific to microbiotas with different composition.

5.5 References

[1] B. Kumar, N. Bhardwaj, K. Agrawal, V. Chaturvedi, P. Verma, Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept, Fuel Process, Technol. 199 (2020), 106244, https://doi.org/10.1016/j.fuproc.2019.106244.

[2] A. Sanchez, G. Magana, ˜ D. Gomez, M. Solís, R. Banares-Alcantara, Bidimensional sustainability analysis of lignocellulosic ethanol production processes, Method and case study, Biofuels, Bioprod. Bioref. 8 (2014) 670–685, https://doi.org/10.1002/bbb.1512.

[3] G. Centi, S. Perathoner, Chapter 16 - Catalytic Transformation of CO2 to Fuels and Chemicals, with Reference to Biorefineries, in: K.S. Triantafyllidis, A.A. Lappas,

M. Stocker ¨ (Eds.), Eds.), The role of catalysis for the sustainable production of biofuels and bio-chemicals, Elsevier, 2013, pp. 529–555.

[4] A. He, X. Kong, H. C. Wang, M. Wu, J. Jiang, P. Ma, Ouyang, Efficient carbon dioxide utilization and simultaneous hydrogen enrichment from off-gas of acetone–butanol–ethanol fermentation by succinic acid producing Escherichia coli, Bioresour. Technol. 214 (2016) 861–865, https://doi.org/10.1016/j. biortech.2016.04.073.

[5] D. González-Tenorio, K.M. Munoz-Páez, I. Valdez-Vazquez, Butanol production coupled with acidogenesis and CO2 conversion for improved carbon utilization, Biomass Conv. Bioref. (2020), https://doi.org/10.1007/s13399-020-00805-y.

[6] M. Kumar, S. Sundaram, E. Gnansounou, C. Larroche, I.S. Thakur, Carbon dioxide capture, storage and production of biofuel and biomaterials by bacteria: A review, Bioresour. Technol. 247 (2018) 1059–1068, https://doi.org/10.1016/j. biortech.2017.09.050.

[7] S.W. Ragsdale, E. Pierce, Acetogenesis and the Wood–Ljungdahl pathway of CO2 fixation, Biochim Biophys Acta Proteins Proteom 1784 (12) (2008) 1873–1898, https://doi.org/10.1016/j.bbapap.2008.08.012.

[8] D. Arslan, K.J.J. Steinbusch, L. Diels, H. De Wever, C.J.N. Buisman, H.V. M. Hamelers, Effect of hydrogen and carbon dioxide on carboxylic acids patterns in mixed culture fermentation, Bioresour Technol. 118 (2012) 227–234, https://doi.org/10.1016/j.biortech.2012.05.003.

[9] P. Darvekar, C. Liang, M.N. Karim, M.T. Holtzapple, Effect of headspace gas composition on carboxylates production in open-culture fermentation of corn stover, Biomass Bioenergy 126 (2019) 57–61, https://doi.org/10.1016/j. biombioe.2019.04.019.

[10] F. Oswald, I.K. Stoll, M. Zwick, S. Herbig, J. Sauer, N. Boukis, A. Neumann, Formic acid formation by Clostridium ljungdahlii at elevated pressures of carbon dioxide and hydrogen, Front. Bioeng. Biotechnol. 6 (2018) 6, https://doi.org/10.3389/fbioe.2018.00006.

[11] J.A. Modestra, R. Katakojwala, M. Venkata, CO2 fermentation to short chain fatty acids using selectively enriched chemolithoautotrophic acetogenic bacteria, Chem. Eng. J. 394 (2020), 124759, https://doi.org/10.1016/j.cej.2020.124759.

[12] D. Vasudevan, H. Richter, L.T. Angenent, Upgrading dilute ethanol from syngas fermentation to n-caproate with reactor microbiomes, Bioresour. Technol. 151 (2014) 378–382, https://doi.org/10.1016/j.biortech.2013.09.105.

[13] L.A. Kucek, C.M. Spirito, L.T. Angenent, High n-caprylate productivities and specificities from dilute ethanol and acetate: chain elongation with microbiomes to upgrade products from syngas fermentation, Energy Environ, Sci. 9 (2016) 3482–3494, https://doi.org/10.1039/C6EE01487A.

[14] Q. Wang, P. Zhang, S. Bao, J. Liang, Y. Wu, N. Chen, S. Wang, Y. Cai, Chain elongation performances with anaerobic fermentation liquid from sewage sludge with high total solid

as electron acceptor, Bioresour. Technol. 306 (2020), 123188, https://doi.org/10.1016/j.biortech.2020.123188.

[15] I. Valdez-Vazquez, F. Alatriste-Mondragón, J. Arreola-Vargas, G. Buitrón, J. Carrillo-Reyes, E. Leon-Becerril, ´ H.O. Mendez-Acosta, B. Weber, A comparison of biological, enzymatic, chemical and hydrothermal pretreatments for producing biomethane from Agave bagasse, Ind. Crops Prod. 145 (2020), https://doi.org/10.1016/j.indcrop.2020.112160.

[16] K.M. Munoz-Páez, E.L. Alvarado-Michi, G. Buitrón, I. Valdez-Vazquez, Distinct effects of furfural, hydroxymethylfurfural and its mixtures on dark fermentation hydrogen production and microbial structure of a mixed culture, Int. J. Hydrog. Energy 44 (4) (2019) 2289–2297, https://doi.org/10.1016/j.ijhydene.2018.04.139.

[17] K.M. Muñoz-Páez, E.L. Alvarado-Michi, I. Moreno-Andrade, G. Buitrón I. ValdezVazquez, Comparison of suspended and granular cell anaerobic bioreactors for hydrogen production from acid agave bagasse hydrolyzates, Int. J. Hydrog. Energy 45 (1) (2020) 275–285, https://doi.org/10.1016/j.ijhydene.2019.10.232.

[18] Ø. Hammer, D.A.T. Harper, P.D. Ryan, PAST: paleontological statistics software package for education and data analysis, Palaeontologia Electronica 4 (2001) 9. Htt p://folk.uio.no/ohammer/past/.

[19] S.K. Butti, J.A. Modestra, S.V. Mohan, S. Varjani, A. Pandey, Chapter 5.5 -Regulating bioelectrochemical CO2 sequestration for platform chemicals production, in: S.V. Mohan (Ed.), Eds.), In Biomass, Biofuels and Biochemicals, Microbial Electrochemical Technology, Elsevier, 2019, pp. 797–824.

[20] N. Fernández, R. Sierra-Alvarez, R. Amils, J.A. Field, J.L. Sanz, Compared microbiology of granular sludge under autotrophic, mixotrophic and heterotrophic denitrification conditions, Water Sci Technol. 59 (6) (2009) 1227–1236, https://doi.org/10.2166/wst.2009.092.

[21] J. Hu, L. Wang, S. Zhang, X. Xi, Y. Le, X. Fu, Y. Tsang, M. Gao, Interactions between autotrophic and heterotrophic strains improve CO_2 fixing efficiency of nonphotosynthetic

microbial communities, Appl Biochem Biotechnol. 176 (5) (2015) 1459–1471, https://doi.org/10.1007/s12010-015-1657-4.

[22] N. Valdes, P. Soto, L. Cottet, P. Alarcon, A. Gonzalez, A. Castillo, G. Corsini, M. Tello, Draft genome sequence of Janthinobacterium lividum strain MTR reveals its mechanism of capnophilic behavior, Stand Genomic Sci 10 (2015) 110, https://doi.org/10.1186/s40793-015-0104-z.

[23] S. Hong, J. Kim, S. Lee, Y.H. In, S.S. Choi, J.K. Rih, J.K. Rih, C.H. Kim, H. Jeong, C. G. Hur, J.J. Kim, The genome sequence of the capnophilic rumen bacterium Mannheimia succiniciproducens, Nat. Biotechnol. 22 (2004) 1275–1281, https://doi.org/10.1038/nbt1010.

[24] H. Zhang, M.A. Bruns, B.E. Logan, Perchlorate reduction by a novel chemolithoautotrophic, hydrogen-oxidizing bacterium, Environ. Microbiol. 4 (10) (2002) 570–576, https://doi.org/10.1046/j.1462-2920.2002.00338.x.

[25] M.C. Vidal, J.J. Cazzulo, CO2 -fixing enzymes in a marine psychophile, J Bacteriol. 112 (1) (1972) 427–433. https://10.0000/JB.asm.org/JB/112/1/427.

[26] J.R. Leadbetter, T.M. Shmidt, J.R. Graber, J.A. Breznak, Acetogenesis from H2 plus CO2 by Spirochetes from Termite Gut, Science 283 (5402) (1999) 686–689, https://doi.org/10.1126/science.283.5402.686. [27] S. O-Thong, X. Zhu, I. Angelidaki, S. Zhang, [27] G. Luo, Chapter Two - Medium chain fatty acids production by microbial chain elongation: Recent advances, in: Y. Li, S.K. Khanal (Eds.), (Eds.), Advances in Bioenergy, 5, Elsevier, 2020, pp. 63–99.

[28] K.D. de Leeuw, C.J.N. Buisman, D.P.B.T.B. Strik, Branched medium chain fatty acids: iso-caproate formation from iso-butyrate broadens the product spectrum formicrobial chain elongation, Environ. Sci. Technol 53 (13) (2019) 7704–7713, https://doi.org/10.1021/acs.est.8b07256.

[29] M. Lin, X. Dai, P.J. Weimer, Shifts in fermentation end products and bacterial community composition in long-term, sequentially transferred in vitro ruminal enrichment cultures fed switchgrass with and without ethanol as a co-substrate, Bioresour. Technol. 285 (2019), 121324, https://doi.org/10.1016/j. biortech.2019.121324.

[30] R. Slezak, J. Grzelak, L. Krzystek, S. Ledakowicz, The effect of initial organic load of the kitchen waste on the production of VFA and H2 in dark fermentation, Waste Manage. 68 (2017) 610–617, https://doi.org/10.1016/j.wasman.2017.06.024.

[31] J.N. Sunarno, P. Prasertsan, W. Duangsuwan, B. Cheirsilp, K. Sangkharak, Biodiesel derived crude glycerol and tuna condensate as an alternative low-cost fermentation medium for ethanol production by Enterobacter aerogenes, Ind. Crops Prod. 138 (2019), 111451, https://doi.org/10.1016/j.indcrop.2019.06.014.

[32] C.H. Lay, H.C. Lin, B. Sen, C.Y. Chu, C.Y. Lin, Simultaneous hydrogen and etanol production from sweet potato via dark fermentation, J. Cleaner Prod. 27 (2012) 155–164, https://doi.org/10.1016/j.jclepro.2011.12.027.

[33] J. Wiegel, Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes, Experientia. 36 (1980) 1434–1446, https://doi.org/10.1007/BF01960144.

[34] L. Liu, J.Y. Jiao, B.Z. Fang, A.P. Lv, Y.Z. Ming, M.M. Li, N. Salam, W.J. Li, Isolation of Clostridium from Yunnan-Tibet hot springs and description of Clostridium thermarum sp. nov. with lignocellulosic ethanol production, Syst. Appl. Microbiol. 43 (5) (2020), 126104, https://doi.org/10.1016/j.syapm.2020.126104.

[35] K. Arslan, B. Bayar, H.N. Abubackar, M.C. Veiga, C. Kennes, Solventogenesis in Clostridium aceticum producing high concentrations of ethanol from syngas, Rioresour. Technol. 292 (2019), 121941, https://doi.org/10.1016/j.biortech.2019.121941.

[36] M.U. Monir, A.A. Aziz, F. Khatun, A. Yousuf, Bioethanol production through syngas fermentation in a tar free bioreactor using Clostridium butyricum, Renew. Energy. 157 (2020) 1116–1123, https://doi.org/10.1016/j.renene.2020.05.099.

[37] N. Reichardt, M. Vollmer, G. Holtrop, et al., Specific substrate-driven changes in human faecal microbiota composition contrast with functional redundancy in short-chain fatty acid production, ISME J. 12 (2) (2018) 610–622, https://doi.org/10.1038/ismej.2017.196.

Capítulo 6 Resultados. Análisis de sustentabilidad de biorrefinerías lignocelulósicas mono y multiproducto con biofijación de CO_2.

El Capítulo 6 estuvo dedicado al cumplimiento del objetivo específico 5. Para ello, se trabajó en la modelación y análisis de biorrefinerías para la producción de butanol y otros productos, que incluyen la fijación biológica de CO_2, la modelación fue alimentada con los resultados experimentales obtenidos previamente. De los resultados del objetivo específico 1 sobre la fermentación acidogénica (Capítulo 3) se consideró datos de la productividad de los gases (H_2/CO_2), la concentración de los AGV (acetato y butirato) y las condiciones de operación de la fermentación acidogénica. De los resultados del objetivo específico 2 sobre la fermentación ABE (Capítulo 3), se utilizaron las condiciones de operación de la fermentación ABE. De los resultados del objetivo específico 3 sobre el incremento de la producción de butanol (Capítulo 4) se empleó la concentración de butanol lograda por la evolución adaptativa en laboratorio. Respecto a los resultados del objetivo específico 4 sobre la biofijación de CO_2 a través de la fermentación de gases (Capítulo 5), solo se considera la prueba de concepto de una biorrefinería con biofijación de CO_2 para obtener caproato, un producto de alto valor económico.

La motivación del trabajo fue establecer diferentes modelos de biorrefinería basadas en los bioprocesos en cascada estudiados, y determinar qué modelo es más sustentable. Los modelos de biorrefinería estudiados fueron: 1) el modelo base (MB) que integra la fermentación acidogénica, la fermentación ABE, la digestión anaerobia, cogeneración y purificación de productos siendo los principales productos el butanol, calor y electricidad; 2) el modelo con biofijación de CO_2 que tiene los mismos bioprocesos del MB y además la fermentación de gases provenientes de la acidogénesis para la producción adicional de butanol (modelo con 2 escenarios, MCO_2ButOH-I y MCO_2ButOH-II); y 3) el modelo con biofijación de CO_2 con la misma configuración que el modelo 2, con la diferencia de que la fermentación de gases produce caproato (modelo con 1 escenario, MCO_2Cap).

La estrategia fue emplear los resultados de los cuatro objetivos específicos previos para alimentar la simulación de las biorrefinerías en el software SuperPro Designer v11 (SPD) considerando una capacidad de alimentación del sustrato de 1000 toneladas de residuos de

maíz por año. Después, los resultados que se obtuvieron de la simulación se emplearon junto con ecuaciones reportadas en la literatura para la evaluación de dos indicadores económicos (eficiencia de la biorrefinería y costo total de producción, CTP); tres indicadores ambientales (consumo de agua, gases de efecto invernadero emitidos, calidad de agua residual y relación de la energía empleada y consumida) y un indicador social (número de empleos).

Después, estos indicadores son monetizados empleando la unidad funcional de USD por tonelada de sustrato y conformando un indicador global para la comparación entre los escenarios. También se realizó una asignación económica al CTP. Del análisis técnico-económico los resultados fueron un CTP de 1.23 USD/$kg_{butanol}$ en BM. En MCO_2ButOH-II, la conversión de CO_2 en butanol adicional resulta en un CTP de 1.21 USD/$kg_{butanol}$, por lo que, la conversión del CO_2 en butanol adicional no disminuye los costos de forma notable. A diferencia, en MCO_2Cap, el CTP del butanol se redujo a 0.18 USD/$kg_{butanol}$, esto porque la mayor parte del costo de producción se atribuye al caproato.

Respecto al análisis de sustentabilidad, el desempeño ambiental de MCO_2ButOH-II es mejor que el desempeño ambiental de MB, esto porque la biofijación de CO_2 en butanol, se refleja en una mayor eficiencia energética, junto con un menor consumo de agua y menor contaminación en el agua residual. En el dominio económico, los altos costos del proceso de biofijación de CO_2 para producir caproato en MCO_2Cap tienen un impacto negativo en la sustentabilidad. También, los bajos rendimientos de la transformación de azúcares en la producción de productos en MB y MCO_2ButOH-I son impactos negativos sobre el indicador global. En el dominio social, MCO_2ButOH-I y MCO_2Cap tuvieron una mejora por el proceso adicional y mayores flujos de operación, por lo que son beneficios en la sustentabilidad.

Finalmente, se estableció que la biorrefinería virtual conformada por los bioprocesos en cascada de fermentación acidogénica, fermentación ABE, y biofijación de CO_2 para la producción solo de butanol es más sustentable que los otros modelos, debido principalmente a un superior desempeño ambiental.

Referencia del trabajo sometido para publicación

González-Tenorio D, Sanchez A, Patricia Güereca LP, Valdez-Vazquez I. 2021.
Sustainability analysis of single- and multi-product lignocellulosic biorefineries using biological CO_2 fixation.
Journal of CO2 Utilization.

6.1 Abstract

This work analyzes the sustainability performance of single- and multi-product lignocellulosic biorefineries that use carbon dioxide (CO_2) fixation to produce butanol, a promising biofuel alternative, and a high-value product such as caproate. Three different biorefinery models are evaluated in this work: 1) the base model (BM) which produces mainly butanol and includes biomass milling, acidogenesis, acetone-butanol-ethanol (ABE) fermentation, anaerobic digestion, cogeneration, and purification of products; 2) a biorefinery model which uses CO_2 biofixation for additional butanol production (MCO_2ButOH) by either using H_2/CO_2 from acidogenesis (MCO_2ButOH-I), or H_2/CO_2 and short-chain fatty acids (MCO_2ButOH-II); and 3) a biorefinery model using CO_2 biofixation for caproate production (MCO_2Cap) from available by-products (H_2/CO_2, short-chain fatty acids, and ethanol). The techno-economic analysis of the three biorefinery models was carried out using the SuperPro Designer v11 (SPD) software and considering a processing capacity of 1,000 tons of corn stover/year. The following indicators were evaluated: two economic indicators (biorefinery efficiency and total production cost [TPC]), four environmental indicators (water consumption, greenhouse gas emitted, wastewater quality, and end-use energy ratio), and one social indicator (employment). To allow for a comparison among different scenarios, indicators are monetized using a functional unit of USD/ton of substrate. Economic allocation among co-products resulted in a TPC of 1.23 USD/$kg_{butanol}$ for the BM. In the MCO_2ButOH-II model, the conversion of CO_2 into additional butanol resulted in a TPC of 1.21 USD/$kg_{butanol}$, while in the MCO_2Cap model the conversion of CO_2 into caproate resulted in a TPC of 0.18 USD/$kg_{butanol}$. MCO_2ButOH-II has a better environmental performance than MB, MCO_2ButOH-I, and MCO_2Cap, due primarily to a higher end-use energy ratio. Biorefinery efficiency improves in those models employing CO_2 fixation, especially in the MCO_2ButOH-II model. However, the normalized TPC increases in all models, thus decreasing their global economic performance. Interestingly, additional processes required in the biorefinery models using CO_2 fixation positively affect the social domain.

Moreover, overall sustainability results indicate that coupling CO_2 fixation with biofuel production is more sustainable for single-product biorefineries than multi-product plants because of a better environmental performance.

Keywords: ABE fermentation; caproate; cascading process; lignocellulose; total production cost.

6.2 Introduction

Butanol is an alternative engine fuel which presents several advantages with respect to gasoline [1]. Butanol is a renewable fuel produced in biorefineries, processing facilities that convert biomasses into a variety of marketable bioproducts (food ingredients, chemicals, and materials) and energy (fuels, energy, and heat) [2]. The biochemical pathways that produce butanol include the acetone-butanol-ethanol (ABE) fermentation of sugars and the Wood-Ljungdahl pathway from C1-carbon sources or gas fermentation [3]. Recently, several authors have coupled these two bioprocesses to maximize product yield [3-6]. For example, González-Tenorio and colleagues. [3], presented a biorefinery cascade processing that integrates acidogenesis, ABE fermentation and gas fermentation to produce butanol from sugars and fermenter off-gases. Technology selection and biorefinery configuration are influenced by economic criteria (i.e., total production costs [TPC], environmental criteria (i.e., greenhouse gas [GHG] emissions, water usage, wastewater pollution, and energy use [7], and social criteria (i.e., number of jobs created) [8,9].

Most literature reports the techno-economic analysis (TEA) of lignocellulosic biorefineries for the production of butanol via ABE fermentation [10-14]. Qureshi et al. [10] reported a TPC of 1.23 USD/$kg_{butanol}$ when butanol was produced from wheat straw using traditional technologies including dilute acid pretreatment, separate hydrolysis and fermentation, and distillated recovery. The TPC reduced to 1.20 USD/$kg_{butanol}$ when substrate cost decreased, and to 1.0 USD/$kg_{butanol}$ when the purification technology was changed (recovery by membrane instead of distillation). In 2016, Later on, Baral & Shah [11] reported a TPC between 1.9 and 2.2 USD/$kg_{butanol}$ for corn waste processing by chemical pre-treatment, detoxification, and simultaneous saccharification and fermentation. Sanchez et al. [13] proposed a biorefinery cascade using consolidated bioprocessing (CBP). This study evaluated the impact of the biorefinery processing capacity on TPC, ranging from 0.9 USD/$kg_{butanol}$ (smallest capacity, 100 ton/d) to 0.8 USD/$kg_{butanol}$ (largest capacity, 1000 ton/d). More recently, Valdez-Vazquez &

Sanchez [14] analyzed the impact of fermentation time and product concentration on TPC in a CBP-based biorefinery. TPC values ranged from 1.2 to 1.6 USD/$kg_{butanol}$ with product concentration having a greater effect than fermentation time.

In terms of environmental impact, previous studies have only quantified CO_2 emissions and water consumption in biorefineries producing butanol via ABE fermentation. For example, Brito & Martin [15] conducted a life cycle analysis (LCA) of a traditional biorefinery producing butanol from wheat straw with acid pre-treatment, enzymatic hydrolysis, and ABE fermentation. They obtained a value of 2 756 108 $gCO_2/ton_{substrate}$ and of 28 996 $L/ton_{substrate}$ for CO_2 emissions and water consumption, respectively.

So far, the advantages of coupling CO_2 fixation to single- or multi-product biorefinery value chains to produce butanol from lignocellulosic biomass have not been analyzed. Therefore, this work analyzes the sustainability performance of a CBP-based, butanol-producing biorefinery coupled to CO_2 biofixation, considering three models: 1) a base model (BM) using consolidated bioprocessing to convert corn stover into butanol (Figure 1A); 2) a biorefinery model that implements biological CO_2 biofixation for the production of additional butanol with two scenarios (MCO_2ButOH-I and MCO_2ButOH-II, Figure 1B and Figure 1C, respectively); and 3) a biorefinery model that uses CO_2 biofixation to produce caproate with a single scenario (MCO_2Cap, Figure 1D). The sustainability analysis includes economic, environmental, and social indicators for the abovementioned biorefinery models.

6.3 Description of biorefinery models analyzed

This study evaluates three biorefinery models that receive 1000 ton/d of corn stover with the following composition (% in dry matter): 23.5% soluble compounds, 37.9% cellulose, 27.9% hemicellulose, 2.8% lignin, and 1.8% ashes [3].

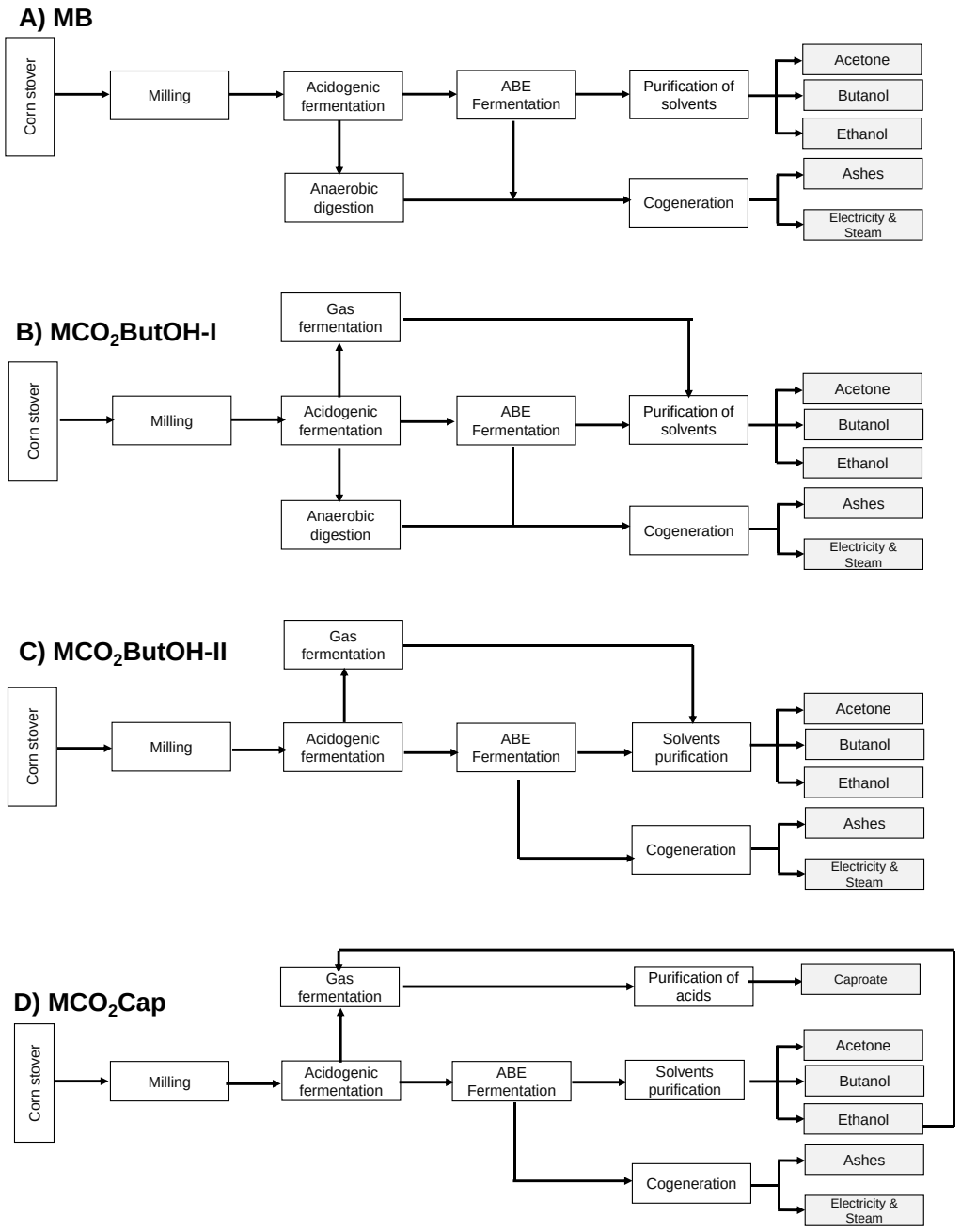

Figure 6.1. Block diagram of the biorefinery processes for butanol production. A) Base model (BM). B) Model coupled with biological CO_2 fixation for butanol production, scenario I (MCO_2ButOH-I). C) Model coupled with biological CO_2 fixation for butanol production, scenario II (MCO_2ButOH-I). D) Model coupled with biological CO_2 fixation for caproate production (MCO_2Cap).

6.3.1 Base model (BM)

The base model (BM) is based on a biorefinery cascade processing that uses CBP mediated by microbial consortia with six main stages: i) milling, ii) acidogenic fermentation, iii) ABE

fermentation, iv) anaerobic digestion, v) solvent separation, and vi) electricity-steam cogeneration ([13,14]; Figure 1A). The BM produces butanol, acetone, ethanol, electricity, and steam.

6.3.1.1 Milling

The biorefinery receives corn stover to be processed in three crushers to reduce the particle size to 3 ± mm (Figure B1, Appendix B). Subsequently, the raw material is sent to the acidogenic fermentation.

The first CBP consists of an acidogenic fermentation mediated by the native microbial consortium of the substrate that converts the soluble and xylan fractions into H_2/CO_2, acetate, and butyrate [3]. This stage considers experimental data according to the conversion percentages shown in Table A1, Appendix A. For details about reactor operation see Figure B2, Appendix B. The digestate (cells and cellulose/lignin/ashes) is sent to the ABE fermentation, acetate/butyrate are sent to anaerobic digestion, and H_2/CO_2 are sent to cogeneration.

6.3.1.2 Acidogenic fermentation

The first CBP consists of an acidogenic fermentation mediated by the native microbial consortium of the substrate that converts the soluble and xylan fractions into H_2/CO_2, acetate, and butyrate [3]. This stage considers experimental data according to the conversion percentages shown in Table A1, Appendix A. For details about reactor operation see Figure B2, Appendix B. The digestate (cells and cellulose/lignin/ashes) is sent to the ABE fermentation, acetate/butyrate are sent to anaerobic digestion, and H_2/CO_2 are sent to cogeneration.

6.3.1.3 ABE Fermentation

The second CBP consists of ABE fermentation mediated by a microbial consortium producer of butanol that converts cellulose into ABE [16]. The simulation of this stage considers experimental data according to the conversion percentages shown in Table A2, Appendix A. For details about reactor operation Figure B3, Appendix B. The outlet stream is divided into two streams: the solvents are sent to separation and the solids (mainly lignin and ashes) are sent to cogeneration. The CO_2 produced is vented to the atmosphere.

6.3.1.4 Anaerobic digestion

The third bioprocess consists of an anaerobic digestion that receives acetate/butyrate from acidogenesis converting them into CH_4, CO_2, and cells according to reactions and conversion

percentages shown in Table A3, Appendix A [17]. For details about reactor operation see Figure B4, Appendix B. The gaseous stream is sent to cogeneration. The effluents from this process contain mostly water (99.5%) are recirculated to the acidogenic fermentation providing 91.5% of the water required (Figure C1, Appendix C).

6.3.1.5 Solvent separation

This stage receives the liquid stream from ABE fermentation. A hybrid separation system purifies butanol according to the previously reported configuration [13, 14]; see Figure B5, Appendix B). The distillation produces 99.7% butanol, 99.9% acetone, and 75% ethanol.

6.3.1.6 Electricity-steam cogeneration

This stage receives H_2/CO_2 from acidogenesis, CH_4/CO_2 from anaerobic digestion, and solids from ABE fermentation to generate steam and electricity used in the biorefinery (Figure B6, Appendix B).

6.3.2 Model coupled with biological CO_2 fixation for butanol production (MCO_2ButOH)

This biorefinery model couples the biological CO_2 fixation for butanol production (MCO_2ButOH) via the fermentation of off-gases and acetate/butyrate produced in acidogenesis [3]. MCO_2ButOH consists of six main stages: i) milling, ii) acidogenic fermentation, iii) ABE fermentation, iv) CO_2 fixation (gas fermentation), v) solvent separation, and vi) electricity-steam cogeneration. MCO_2ButOH has two scenarios based on substrates available in acidogenesis: scenario I (MCO_2ButOH-I) considers only H_2 and CO_2 (Figure 1B) while scenario II (MCO_2ButOH-II) considers H_2, CO_2, and acetate/butyrate (Figure 1C). MCO_2ButOH produces butanol, acetone, ethanol, electricity, and steam.

The stage of milling, acidogenic fermentation and ABE fermentation, remain the same as the BM described previously in Sections 2.1.1, 2.1.2, and 2.1.3, respectively. MCO_2ButOH-I omits the anaerobic digestion process present in BM.

6.3.2.1 Gas fermentation for butanol production, scenario I (MCO_2ButOH-I)

MCO_2ButOH-I considers gas fermentation of off-gases of acidogenesis to produce butanol, while acetate/butyrate are sent to anaerobic digestion (same as BM). MCO_2ButOH-I assumes the theoretical yields (Table A4, Appendix A) to elucidate the maximum potential of gas fermentation for converting the substrates into butanol (Figure C2, Appendix C). For details about reactor operation see Figure B7a, Appendix B. The stream containing butanol is sent to the separation stage together with ABE products.

6.3.2.2 Solvent separation

This stage receives a 2.2 times higher flow than BM from ABE fermentation (13.8 g/$L_{butanol}$) and gas fermentation (2.6 g/$L_{butanol}$) with a final concentration of 16.4 g/$L_{butanol}$. The effluents from this process contain mostly water (99.5%) that are recirculated to the acidogenic fermentation providing 68.6% of the water required (Figure C1, Appendix C).

6.3.2.3 Gas fermentation for butanol production, scenario II (MCO_2ButOH-II)

MCO_2ButOH-II considers gas fermentation of off-gases of acidogenesis together with acetate/butyrate to produce butanol. In consequence, MCO_2ButOH-II omits anaerobic digestion. MCO_2ButOH-II assumes the theoretical yields (Table A4, Appendix A) to elucidate the maximum potential of gas fermentation for converting the substrates into butanol (Figure C2, Appendix C). For details about reactor operation see Figure B7b, Appendix B. The stream containing butanol is sent to the separation stage together with ABE products.

6.3.2.4 Solvent separation

This stage receives a 2.5 times higher flow than BM from ABE fermentation (13.8 g/$L_{butanol}$) and gas fermentation (9.2 g/$L_{butanol}$) with a final concentration of 23.0 g/$L_{butanol}$. The effluents from this process contain mostly water (99.7%) that are recirculated to the acidogenic fermentation providing 95.4% of the water required (Figure C1, Appendix C).

6.3.2.5 Electricity-steam cogeneration

This stage receives CH_4/CO_2 from anaerobic digestion (only for MCO_2ButOH-I) and solids from ABE fermentation to generate steam and electricity used in the biorefinery. The steam generator operates at 86 bar and 510 °C, the steam powers an expansion turbine generating steam at low and high pressure [14].

6.3.3 Model coupled with biological CO_2 fixation for caproate production (MCO_2Cap)

This biorefinery model explores the potential of gas fermentation to produce caproate (MCO_2Cap) via the fermentation of off-gases and acetate/butyrate produced in acidogenesis together with ethanol produced in ABE fermentation [18]. MCO_2Cap consists of seven main stages: i) milling, ii) acidogenic fermentation, iii) ABE fermentation, iv) CO_2 fixation **(**gas fermentation), v) solvent separation, vi) carboxylic acid purification, and vii) electricity-steam cogeneration (Figure 1D). MCO_2Cap produces acetic acid, butanol, acetone, caproate, electricity, and steam.

The stages of milling, acidogenic fermentation, ABE fermentation, and solvent separation remain the same as the BM described previously in Sections 2.1.1, 2.1.2, 2.1.3, and 2.1.5, respectively. MCO_2Cap omits the anaerobic digestion process present in BM.

6.3.3.1 Gas fermentation for caproate production (MCO_2Cap)

MCO_2Cap considers gas fermentation of off-gases of acidogenesis to produce ethanol, then, together with ethanol produced in ABE fermentation, it serves as electron donor to condense acetate/butyrate into caproate [18]. MCO_2Cap assumes the theoretical yields (Table A5, Appendix A) to elucidate the maximum potential of gas fermentation for converting the substrates into caproate. For details about reactor operation see Figure B8, Appendix B. The stream containing caproate is sent to an independent purification stage.

6.3.3.2 Carboxylic acid purification

This stage receives the liquid stream from gas fermentation. The mass flow represents 310 725 kg/h with a concentration of 8.3 $g/L_{caproate}$. The purification train consisted of two treatments [19], the first treatment uses an extraction column using 10% alamina 336 as an extractive agent and the second treatment consist of one distillation column at pressure of 1.0 bar that produces 98.5% caproate. The effluents from this process contain mostly water (47.7%) and traces of acetate, biomass and caproate that are recirculated to the acidogenic fermentation process, providing 95.4% of the required water (Figure C1, Appendix C).

6.3.3.3 Electricity-steam cogeneration

This stage cogeneration receives solids from ABE fermentation to generate electricity-steam used in the operation of the same biorefinery, and this stage is similar to the model described by Sánchez et al. [17].

6.3.4 Techno-economic analysis

The steady-state mass and energy balances for each biorefinery model are solved using the SuperPro Designer® Simulator (SPD) v11.0 (Intelligen, Inc., NJ, USA). Regarding the energy balance, the maximum energy recovery is considered by means of Pinch Point Analysis, with this analysis there is an optimal heat exchange between process streams [20]. Then, the energy efficiency ratio (EER) is determined, which was defined as the ratio between energy production (steam, energy, and chemical energy from solvents and products) and energy demand (heating, cooling, and electricity), as previously described by Sanchez et al. [17].

Economic allocation serves for the calculation of TPC. The allocation is used for the calculation of TPC co-products. The allocation describes as the distribution of the inflows and outflows

among the products obtained throughout the system [21]. Among the types of allocation are mass, energy, and economic. In biorefineries is recommended to use the economic allocation or by energy [22]. In this work, economic allocation is selected because caproate is not an energy product, it is used as a raw material for other products. The economic allocation distributes among all output co-products based on the percentage contribution of each co-product on the total revenues [22]. The DCF is calculated as taxes, financial costs, fixed capital, borrowing annuity, direct production costs (raw materials, supplies, labor, services, and maintenance cost). These costs are obtained from a correlation based on equipment costs. The equipment cost values are obtained from the SPD database. All costs are for the year 2021. The DCF calculates the TPC, Initial investment (INV), and NPV based on these data. The operating costs (OC) consider raw materials, supplies, services, and maintenance.

The following parameters are considered for the TPC: 330 days of operation per year, percentage of leverage (30% of the project), the useful life of plant of 15 years, construction time of 3 years, linear depreciation of equipment in 10 years and with an inflation of 2% [17] at an interest rate of 4%, the percentage presented comes from the interest value reported by The Bank of Mexico in the first half of 2021 (Banxico, 2021).The cost of raw materials considered is 40 USD/ton [25], mesitylene 0.13 USD/kg [14], alamina 0.012 USD/kg, urea 0.210 USD/kg, and $NaHCO_3$ 0.315 USD/kg ([26], consulted on February 10, 2021). The selling price of products are acetone 1.05 USD/kg, n-butanol 1.21 USD/kg, ethanol 0.621 USD/kg [27], and caproate 10 USD/kg ([28], consulted on April 10, 2021).

6.3.5 Sustainability analysis

The sustainability analysis integrates a balance of the three dimensions of sustainability: economic, environmental, and social domains. These analyses are essential in current practices for the biorefinery design to identify improvements in field, however, these studies do not include social impacts which are needed for sustainability assessments [29].

In this study, the sustainability analysis is based on Sanchez et al. [17] according to the following steps: a) the limits, inputs and outputs of the system are identified, as well as the physical limits; b) generators of internal impacts, that is, process activities that cause the impact; c) recipients of external impact, groups or social entities that are affected by internal impacts; d) the problems caused by damage to the recipients of the external impact; e) indicators and f) metrics. The indicators are numerical values associated with impacts, and the metrics are quantifiers of indicators [17]. The metrics are obtained from the mass and energy balance for

each model and in function of one ton of corn stover (TCS). The sustainability framework used for this analysis is shown in Table 1.

Table 1. Sustainability framework adapted from López-Ortega et al. [9] **and Sanchez et al.** [17]**.**

Internal impact generators	External impact receivers	Impacts	Indicator	Metrics
Environmental domain				
Fermentation and cogeneration	Population	Global warming due to GHG	Emitted GHG (GHG)	$M_{GHG,\ \frac{g\ CO_{2\ eq}}{TCS}}$
Acidogenesis fermentation, ABE Fermentation, cogeneration,	Local population	Water availability	Water consumption (WC)	$M_{Wc,\ \frac{L_{fresh\ water}}{TCS}}$
Acidogenesis fermentation, ABE Fermentation, co-generation,	Local population Local industry	Water usage	Wastewater quality (WWQ)	$M_{COD,\ \frac{kg\ COD}{TCS}}$
Production process (plant operation)	Local population Plant owners and investors	Process performance and resources usage	End-use energy ratio (EER)	$M_{EER,\ \frac{MJ_{out}}{MJ_{in}}}$
Economic domain				
Production process (plant operation)	Fuel consumers, plant owners	Process performance and resources usage	Total production cost (TPC) Yield (Y)	$M_{TPC,\ \frac{USD}{TCS}}$ $M_{Y,\ \frac{kg_{polisacaridos}}{TCS}}$
Social domain				
Production process (plant operation)	Local population	Society in the workplace	Employment (E_m)	$M_{Em,\ \frac{Employees}{TCS}}$

Notes: GHG, greenhouse gases; TCS, tons of corn stover; COD, chemical oxygen demand.

Comparing the relative impact of each indicator of the biorefinery model is carried out by doing a normalization with respect to BM, which is the simplest model [7]. The general impact of the biorefinery models is integrated of economic, environmental, and social impacts. The general impact is assessed by weighting indicators metrics through monetization. In this monetization, the same relative importance is established for all metrics and indicators. The monetization is expressed in all the indicators in the same monetary unit, called the functional unit [7]. The monetization is presented in USD/TCS, where the determined values are shown in Table 2. This functional unit is chosen because the products include energy as fuel, or multiple products such as caproate [7]. For the construction of the dimensional functions, one of the following three categories was used [34]. The first category is a metric relationship with some service or product with economic value. For example, GHG emissions are expressed in terms of CO_2-mass equivalents, related to currency through carbon bonds, assigning a cost for each unit of mass emitted. The second category are the costs imposed by regulatory bodies on exploited resources or waste discharge, for example, the payment of rights to extract fresh water from a particular body of water. The third category is a combination of elements from both categories. According to Sánchez et al. [34], negative signs are assigned to the indicators as they are considered harmful. For economic and social indicators, the sign depends on its contribution to sustainability. The global indicator is used for quantification. Then, the comparison is made for the choice of the most sustainable biorefinery.

Table 6.2. Coefficients employed in the sustainability analysis adapted from López-Ortega et al. [9] **and Sanchez et al.** [17]**.**

Dimensional factor	Dimensional function	Value	Reference
Environmental domain			
Emitted GHG (GHG)	$-M_{CHG}\,C_{CO_2}$	C_{CO_2} =1.68 x $10^{-4}\,\frac{USD}{g\ CO_{2\ eq}}$	[30]
Water consumption (WC)	$-M_{Wc}C_{WC}$	C_{WC} =4.98 x $10^{-3}\,\frac{USD}{L_{fresh\ water}}$	[31]
Wastewater quality (WWQ)	$-M_{COD}\ \ C_{COD}$	C_{COD}=3.72 x $10^{-1}\,\frac{USD}{kg_{COD}}$	[9]
End-use energy ratio (EER)	$(M_{EER}\ -1)\ (C_{EER}\ )$	C_{EER-BM} =1.7 x 10^2, $C_{EER\ \mathrm{MCO2ButOH-I}}$ = 1.9 x 10^2 $C_{EER\ \mathrm{MCO2ButOH-II}}$ =2.6 x 10^2, $C_{EER\ \mathrm{MCO2Cap}} = 1.1 \text{ x } 10^3\,\frac{USD}{TCS}$	----
Economic domain			
Total production cost (TPC)	$-M_{TPC}$	$\frac{USD}{TCS}$	----
Yield (Y)	$(M_Y - TRS_B)C_Y$	$C_Y = 4.55 \text{ x } 10^{-1}\ \frac{USD}{kg\ TRS}$	[32]
Social domain			
Employment (Em)	$M_{Em}C_{Em}$	C_{Em}=1.66 x $10^4\,\frac{USD}{\mathrm{Employee}}$	[23], [33]

Notes: GHG, greenhouse gases; COD, chemical oxygen demand; C_{WC}, coefficient of water amount; $C_{COD,}$ coefficient is defined as the rate paid for the discharge of wastewater in Mexico; M_{EER}, metric of End-use energy ratio; C_{EER} is defined as the price of the energy surplus or demanded by the biorefinery; M_Y, metric of yield; TRS_B, biomass total reducing sugars; C_Y, feedstock TRS price; M_{Em}, metric of Employment; C_{Em}, Commission of the Mexican Institute of Social Security-National Union of Social Security Workers IMSS-SNTSS

6.4 Results and discussion

TEA and sustainability analysis were performed for the three biorefinery models considering the following scenarios: 1) BM only produces butanol, acetone, and electricity/steam; 2) MCO_2ButOH uses biofixation of CO_2 for additional butanol production (two different scenarios: MCO_2ButOH-I and MCO_2ButOH-II); 3) MCO_2Cap uses the biofixation of CO_2 to produce caproate.

6.4.1 Techno-economic analysis (TEA)

Table 3 shows Net Present Value (NPV), Initial investment (INV), and Operating Costs (OC) values for the three biorefinery models. NPV is negative for BM and MCO_2ButOH, while positive for the MCO_2Cap model, which results a profitable project under the defined economic conditions. INV values are strictly dependent on the additional bioprocess required for CO_2 fixation doubles INV while in MCO_2Cap additional bioreactors, together with the physicochemical treatment trains for caproate purification increases INV of about 3 times. Regarding OC, BM is the cheapest model while MCO_2Cap is the most expensive one (2.1 times more expensive than BM). In all models, these total costs are mainly conditioned by equipment costs (investment, maintenance, and depreciation) (70%) and raw materials (9%).

Table 6.3 Techno-economic analysis results.

Biorefinery models	NPV ($, millions)	INV ($, millions)	OC ($, millions/yr)
BM	-344.913	264.9	82.5
MCO_2ButOH-I	-781.394	547.2	135.9
MCO_2ButOH-II	-544.44	525.8	131.6
MCO_2Cap	166.582	764.6	180.2

NPV, Net Present Value; INV, Initial investment; OC: Operating Costs.

The resulting TPC (USD/$kg_{butanol}$) with economic allocation for the three biorefinery models are shown in Figure 2. TPC of butanol in BM is 1.23 USD/$kg_{butanol}$. Butanol TPC in the MCO_2ButOH-II model shows a 1% reduction because gas fermentation uses all available substrates from acidogenesis for additional butanol production. On the contrary, TPC of butanol in the MCO_2ButOH-I model increases by 1% when only acidogenic off-gases are used for butanol production. MCO_2Cap model results in a significant reduction of butanol TPC, 65% less than in BM, because of economic allocation. Production of caproate from CO_2 positively impacts the TPC of butanol, since most of the TPC (96%) is allocated to the caproate. The TPC of butanol in the MCO_2Cap model is lower compared to other models [10, 13, 14] that do not include the

additional production of high-value products from CO_2. In MCO_2Cap, the caproate production cost with economic allocation is 7.93 USD/kg. TPC of caproate calculated in this study is higher than those reported in literature. Indeed, caproate production costs ranged from 3.8 to 4.4 USD/kg using yogurt residues as substrate [35], while they were 1.6 USD/kg using chain elongation of short-chain fatty acids [36], and 1.9 USD/kg when artichokes were used as substrate [19].

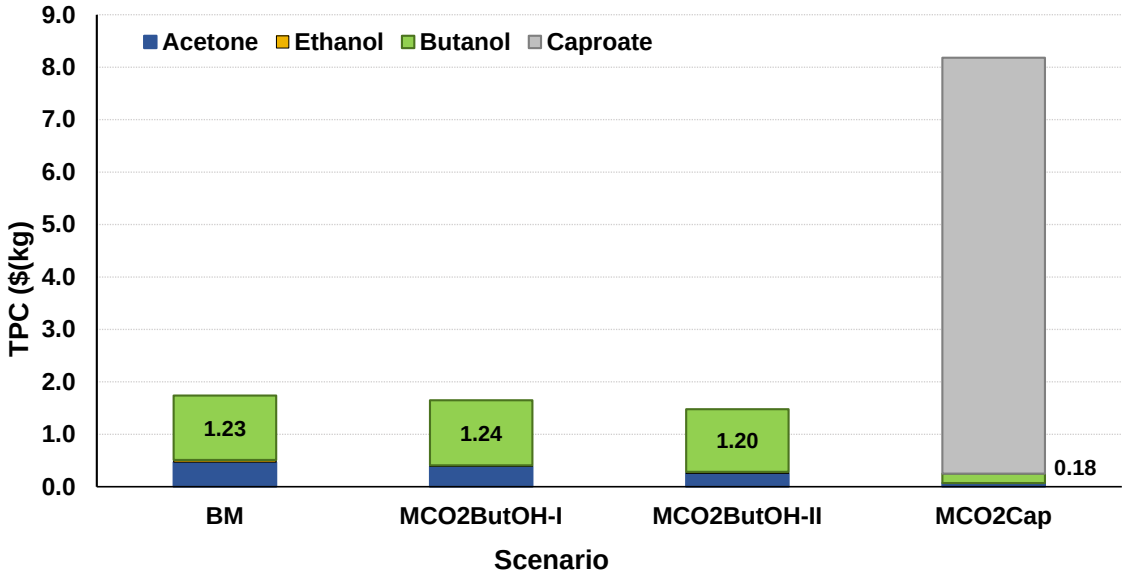

Figure 6.2. Total production cost of products with an economic allocation for three biorefinery models for butanol production. BM, base model. MCO_2ButOH-I, model coupled with biological CO_2 fixation for butanol production, scenario I. MCO_2ButOH-II, model coupled with biological CO_2 fixation for butanol production, scenario II. MCO_2Cap, model coupled with biological CO_2 fixation for caproate production.

6.5 Sustainability analysis

Table 6.4 shows the indicator value and monetized indicator, and Figure 3 shows all normalized economic, environmental, and social indicators for the three biorefinery models.

Table 6.4 Monetized indicator values per domain and global.

Indicator	Indicator value				Monetized indicator $\left(\frac{USD}{TCS}\right)$				Indicator contribution %			
	BM	MCO_2 ButOH-I	MCO_2 ButOH-II	MCO_2 Cap	BM	MCO_2 ButOH-I	MCO_2 ButOH-II	MCO_2 Cap	BM	MCO_2 ButOH-I	MCO_2 ButOH-II	MCO_2 Cap
Environmental Domain												
GHG, $\frac{g\ CO_{2}\ eq}{TCS}$	1.1E+06	9.3E+05	8.4E+05	7.9E+05	-176.49	-156.37	-141.32	-132.49	23.9	21.8	12.9	8.4
W_C, $\frac{L_{fresh\ water}}{TCS}$	8.8E+03	1.4E+04	7.9E+03	1.6E+04	-43.96	-71.55	-39.38	-77.19	6.0	10.0	3.6	4.9
WWQ, $\frac{kg\ COD}{TCS}$	2.5E+01	3.6E+01	1.9E+01	4.2E+01	-9.18	-13.55	-7.12	-15.64	1.2	1.9	0.6	1.0
EER, $\frac{MJ_{out}}{MJ_{in}}$	2.5E+00	2.1E+00	3.4E+00	1.5E+00	253.23	204.17	625.50	556.10	34.3	28.5	56.9	35.1
Total					23.60	-37.31	437.67	330.78	65.5	62.1	74.0	49.3
Economic Indicators												
TPC, $\frac{USD}{TCS}$	1.6E+02	1.9E+02	2.4E+02	7.4E+02	-159.07	-187.09	-242.03	-740.06	21.6	26.1	22.0	46.7
Y, $\frac{USD}{kg\ TRS}$	9.4E+01	1.1E+02	1.5E+02	1.3E+02	-94.13	-82.89	-42.11	-60.85	12.8	11.6	3.8	3.8
Total					-253.20	-269.98	-284.14	-800.92	34.3	37.6	25.9	50.6
Social Indicators												
Em, $\frac{USD}{\text{Employee}}$	8.7E-05	1.0E-04	8.7E-05	1.0E-04	1.45	1.69	1.45	1.69	0.2	0.2	0.1	0.1
Total					1.45	1.69	1.45	1.69	0.2	0.2	0.1	0.1
Global					-228.15	-305.60	154.98	-468.45	100.00	100.00	100.0	100.0

Notes: BM, base model; MCO_2ButOH-I, biorefinery model using CO_2 fixation for butanol production, scenario I; MCO_2ButOH-II, biorefinery model using CO_2 biofixation for butanol production, scenario II; MCO_2Cap, biorefinery model using CO_2 biofixation for caproate production; GHG, greenhouse gases; WC, Water consumption; WWQ, Wastewater quality; EER, End-use energy ratio; TPC, Total production cost; Y, Yield; Em, Employment.

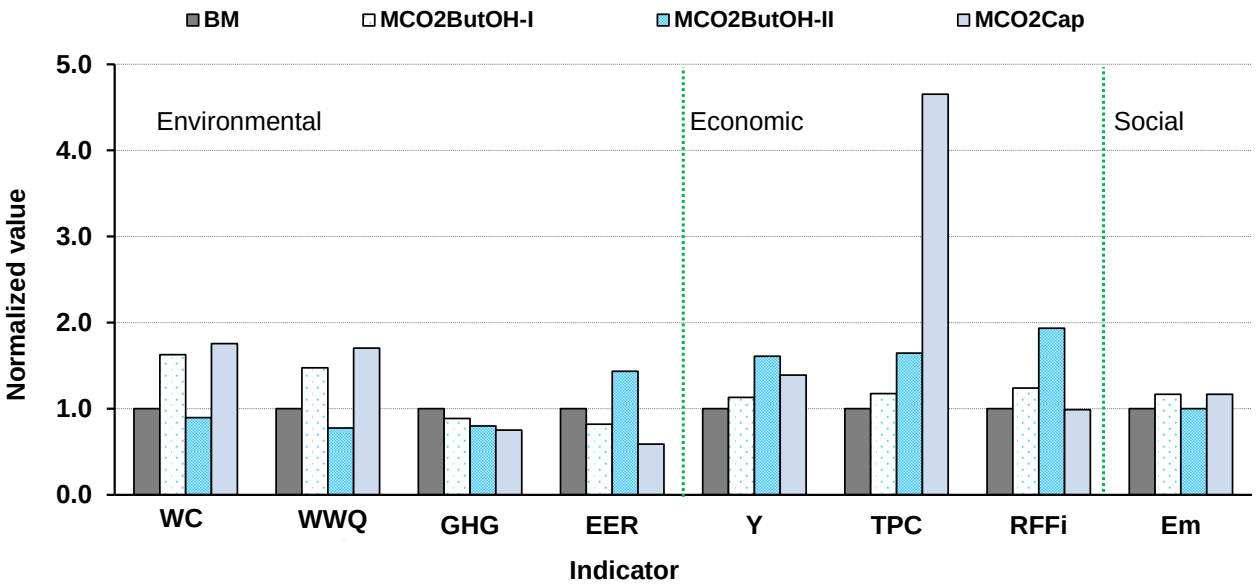

Figure 6.3. Normalized environmental, economic, and social indicators for three biorefinery models for butanol production. BM, base model. MCO_2ButOH-I, model coupled with biological CO_2 fixation for butanol production, scenario I. MCO_2ButOH-II, model coupled with biological CO_2 fixation for butanol production, scenario II. MCO_2Cap, model coupled with biological CO_2 fixation for caproate production. WC, water consumption; WWQ, wastewater quality; GHG, greenhouse gas emissions; EER, energy efficiency ratio; Y, biorefinery efficiency; TPC, total production cost; RFFi, reduction of fossil fuel imports; Em, employment.

6.5.1 Environmental domain

The water consumption (WC) indicator is lower in MCO_2ButOH-II compared to BM since water recirculation provides 95.4% of the total water required for the acidogenic fermentation process; in addition, MCO_2ButOH-II produces a higher amount of butanol with respect to the BM. WC values reported in literature for similar biorefinery models are contrasting. For example, the WC indicator for a traditional lignocellulosic biorefinery with sequential processes is 2,978 $L_{fresh\ water}/ton_{susbstrate}$ [11]. The MCO_2ButOH-II model involving three bioprocesses had a WC value of 7,906 $L_{fresh\ water}/ton_{susbstrate}$ which is substantially lower compared to the WC of a traditional lignocellulosic biorefinery which produces butanol using pretreatment, hydrolysis and ABE fermentation (28,996 $L_{fresh\ water}/ton_{susbstrate}$, [15]).

When the wastewater quality (WWQ) indicator is evaluated, total effluent pollutants are measured as chemical oxygen demand (COD). MCO_2ButOH-I generates a higher COD than MB during the anaerobic digestion process. On the contrary, the MCO_2ButOH-II model has a

lower COD since it uses soluble by-products of acidogenesis in the gas fermentation step. MCO_2ButOH-II reduces COD by using the soluble by-products of acidogenesis and gas fermentation. MCO_2Cap has a higher COD than BM since not all soluble acidogenesis by-products are converted into caproate during gas fermentation because of suboptimal quantities of ethanol. Also, Cavalcante et al. [37] reported that caproate production stops when ethanol is limiting, suggesting the need to estimate the optimal ethanol amount required for the process.

For GHG emissions, both biorefinery models using gas fermentation emit less CO_2 than BM. In other words, when ABE fermentation is used in combination with CO_2 biofixation to produce butanol, total GHG emissions are lower than when ABE fermentation is used alone. The MCO_2Cap model coverts CO_2 into soluble products more efficiently than MCO_2ButOH-I and MCO_2ButOH-II, thus resulting in the biorefinery model with the lowest CO_2 emissions. The MCO_2ButOH-II model (ABE fermentation + CO_2 biofixation) has a GHG emission value of 841,180 $g_{CO2}/ton_{substrate}$ corresponding to a 20% reduction in CO_2 emissions with respect to the BM (1,050,507 $g_{CO2}/ton_{substrate}$). LCA of a lignocellulosic biorefinery producing butanol through sequential processes reported a GHG emission value of 2,756,108 $gCO_2/ton_{substrate}$ [15] corresponding to 3.3 times more emissions compared to the MCO_2ButOH-II model.

Notably, all biorefinery models produce more energy than they consume, with an EER ranging from 1.3 to 3.6. The BM had an EER of 2.5 (normalized to 1.0 in Figure 3). MCO_2ButOH-II had the highest EER (3.6), while MCO_2Cap had the lowest EER (1.5). These differences are due to the fact that MCO_2ButOH-II produces more butanol (53,965 ton/year) than MCO_2Cap (29,856 ton/year). In addition, MCO_2Cap requires more energy than MCO_2ButOH-II for caproate purification. MCO_2ButOH-II EER was substantially higher (38.5%) than that the previously described CBP-based biorefineries not using CO_2 fixation [14], confirming that CO_2 biofixation is a better option in terms of energy recovery than anaerobic digestion.

6.5.2 Economic domain

The yield (Y) indicator quantifies biorefinery efficiency and it is defined as of the organic carbon present in total sugars per ton of substrate [9]. Biorefinery models using gas fermentation can reach Y values up to 61% since they convert more sugars into final products than BM (Y=13%). The MCO_2ButOH-II model has the highest Y because larger amounts of sugars are transformed into products.

Differently, coupling gas fermentation to BM increases the normalized TPC in MCO_2ButOH and MCO_2Cap. ABE fermentation and gas fermentation share the purification stage in MCO_2ButOH

models, but a higher gas flow in the MCO_2ButOH-II fermentation reactor increases the normalized TPC in comparison with the MCO_2ButOH-I model. In MCO_2Cap, the normalized TPC increases considerably because of the additional infrastructures required for gas fermentation and caproate purification.

6.5.3 Social domain

The employment (Em) indicator considers the number of now jobs generated in each biorefinery (considering the process, reactors, type of fluid, and inflow, [23]) by TCS and monetized with the average salary. The C_{Em} coefficient is the average cost per worker based on the salary table of the Commission of the Mexican Institute of Social Security-National Union of Social Security Workers IMSS-SNTSS. MCO_2ButOH and MCO_2Cap biorefinery models generate more jobs than BM due to the presence of additional process units and increased processing capacity. Compared to another fuel production biorefinery that works seasonally but in a high-capacity plant, MCO_2ButOH and MCO_2Cap Em values are lower [9].

6.6 Global sustainability analysis

Figure 4 shows environmental, economic, and social indicators after monetization, as well as the global indicator (i.e., the sum of all indicators). BM, MCO_2ButOH-II, and MCO_2Cap show positive values in the environmental domain (24, 438, and 331 USD/TCS, respectively). MCO_2ButOH-II is the most environmentally friendly model because of the positive contribution of EER (57% of global indicator) in the environmental domain (74%) that compensate for the negative impact of the other indicators such as GHG (12.9%), WC (3.6%), and WWQ (0.6%). In the remaining models, the ERR values also contributes significantly to the environmental domain (Table 4), 29 % and 35 % in MCO_2ButOH-I and MCO_2Cap, respectively.

Regarding the economic domain, all biorefinery models show a negative performance, in particular the MCO_2Cap model. TPC and, to a lesser extent, Y values have a strong negative impact on the global indicator (46.7% and 3.8%, respectively). The three biorefinery models have a positive social impact, especially the MCO_2Cap model, due to the additional stages in the production process (gas fermentation and caproate purification).

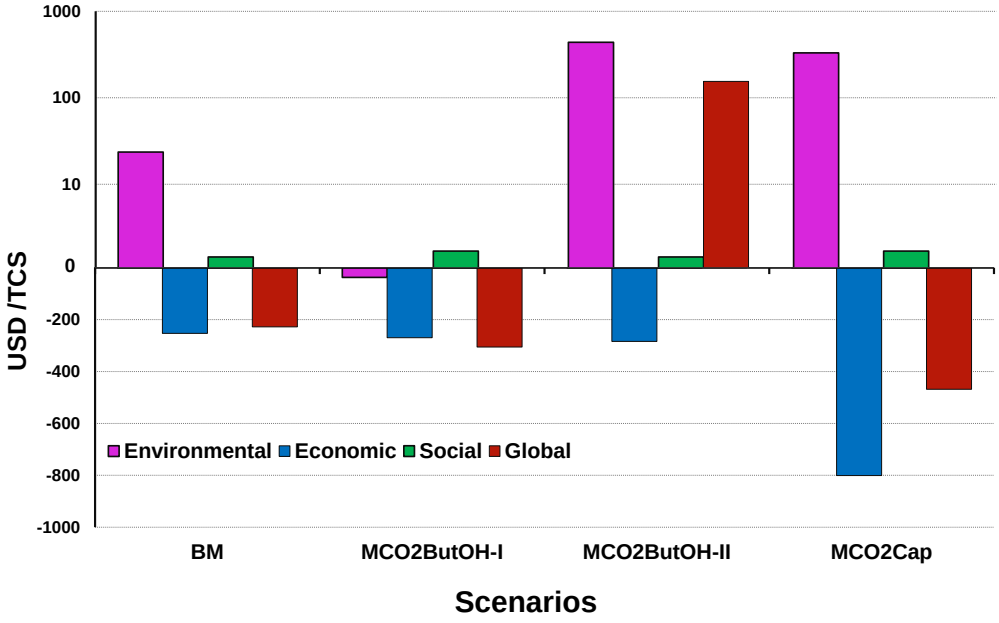

Figure 6.4. Environmental, economic, social, and global indicators after monetization for three biorefinery models for butanol production. BM, base model. MCO_2ButOH-I, model coupled with biological CO_2 fixation for butanol production, scenario I. MCO_2ButOH-II, model coupled with biological CO_2 fixation for butanol production, scenario II. MCO_2Cap, model coupled with biological CO_2 fixation for caproate production.

The global sustainability indicator is positive only in the case of MCO_2ButOH-II. Indeed, in all scenarios, the environmental domain has the biggest impact. In MCO_2ButOH-II, the positive environmental performance counteracts the negative impacts of the economic domain, but in the other models, even if the sum of the environmental indicators is a positive value, it does not to counterbalance the sum of the economic indicators.

6.7 Conclusions

This study evaluates the sustainability performance of single- and multi-product biorefineries using consolidated bioprocessing for butanol production. In addition, we studied the sustainability performance of biorefineries coupling CO_2 biofixation to fermentation to produce higher amounts of butanol or a high-value products such as caproate. Global environmental performance improves significantly when CO_2 is converted to butanol rather than other chemical products, especially because of the contribution of the energy efficiency indicator. However, after monetization, high costs reduce the global economic performance. Biorefinery models using biological CO_2 fixation have a positive social impact due to the employment

generated. However, studies that analyze additional social indicators are required. Compared to BM, MCO2ButOH-II biorefinery model presents several advantages such as reduced water consumption, wastewater pollution, and CO_2 emissions. Despite that, the implementation of a biorefinery with these characteristics is a challenging task. Cascade consolidated bioprocesses mediated by microbial consortia and fixation processes that efficiently convert CO_2 from fermentation off-gases into butanol need to be optimized. The analysis of biorefinery models in this study was performed considering the yield of CO_2 fixation equal to 100%. The biggest challenge will be to reproduce these concentrations experimentally.

6.8 References

[1] N. M Huzir, M. M. A. Aziz, S.B. Ismail, B. Abdullah, N.A. N. Mahmood, N.A. Umor, S.A.F.S. Muhammad, Agro-industrial waste to biobutanol production: Eco-friendly biofuels for next generation, Renew. Sustain. Energy Rev. 94 (2018) 476-485, https://doi.org/10.1016/j.rser.2018.06.036.

[2] G. Dragone, A.A.J. Kerssemakers, J.L.S.P. Driessen, C.K. Yamakawa, L.P. Brumano, S.I. Mussatto, Innovation and strategic orientations for the development of advanced biorefineries, Bioresour Technol. 302 (2020) 122847, https://doi.org/10.1016/j.biortech.2020.122847.

[3] D. González-Tenorio, K.M. Munoz-Páez, I. Valdez-Vazquez, Butanol production coupled with acidogenesis and CO_2 conversion for improved carbon utilization, Biomass Conv. Bioref (2020), https://doi.org/10.1007/s13399-020-00805-y.

[4] G. Lodi, G. De Guido, L. A. Pellegrini, Simulation and energy analysis of the ABE fermentation integrated with gas stripping, Biomass Bioenergy. 116 (2018) 227-235, https://doi.org/10.1016/j.bioBMioe.2018.06.012.

[5] E. Rochón, M. D. Ferrari, C. Lareo, Integrated ABE fermentation-gas stripping process for enhanced butanol production from sugarcane-sweet sorghum juices, Biomass Bioenergy 98 (2017) 153-160, https://doi.org/10.1016/j.bioBMioe.2017.01.011.

[6] A. S. P. Pamula, D. J. Lampert, H. K. Atiyeh, Well-to-wake analysis of switchgrass to jet fuel via a novel co-fermentation of sugars and CO_2, Sci. Total Environ. 782 (2021) 146770, https://doi.org/10.1016/j.scitotenv.2021.146770.

[7] A. Sanchez, O.R. Ayala, P. Hernandez-Sanchez, I. Valdez-Vazquez, A. de León-Rodríguez, An environment-economic analysis of hydrogen production using advanced biorefineries

and its comparison with conventional technologies, Int. J. Hydrog. Energy. 45 (51) (2020) 27994-28006, https://doi.org/10.1016/j.ijhydene.2020.07.135.

[8] N. Martinkus, G. Latta, S. A.M. Rijkhoff, D. Mueller, S. Hoard, D. Sasatani, F. Pierobon, M. Wolcott, A multi-criteria decision support tool for biorefinery siting: Using economic, environmental, and social metrics for a refined siting analysis, Biomass Bioenergy 128 (2019) 105330, https://doi.org/10.1016/j.biombioe.2019.105330.

[9] M. G. López-Ortega, Y. Guadalajara, T. L Junqueira, I.L.M. Sampaio, A. Bonomi, A. Sánchez, Sustainability analysis of bioethanol production in Mexico by a retrofitted sugarcane industry based on the Brazilian expertise, Energy 232 (2021) 121056, https://doi.org/10.1016/j.energy.2021.121056.

[10] N. Qureshi, B.C. Saha, M.A. Cotta, V. Singh, An economic evaluation of biological conversion of wheat straw to butanol: A biofuel, Energy Convers. Manag. 65 (2013) 456-462, https://doi.org/10.1016/j.enconman.2012.09.015.

[11] N.R. Baral, A.Shah, Techno-economic analysis of cellulosic butanol production from corn stover through Acetone–Butanol–Ethanol fermentation, Energy Fuels 30 (7) (2016) 5779–5790, https://doi.org/10.1021/acs.energyfuels.6b00819.

[12] N.R. Baral, A. Shah, Techno-economic analysis of utilization of stillage from a cellulosic biorefinery, Fuel Process. Technol. 166 (2017) 59-68, https://doi.org/10.1016/j.fuproc.2017.05.017.

[13] Sanchez, I. Valdez-Vazquez, A. Soto, S. Sánchez, D. Tavarez, Lignocellulosic n-butanol co-production in an advanced biorefinery using mixed cultures, Biomass Bioenergy 102 (2017) 1-12, https://doi.org/10.1016/j.bioBMioe.2017.03.023.

[14] I. Valdez-Vazquez, A. Sanchez, Proposal for biorefineries based on mixed cultures for lignocellulosic biofuel production: a techno-economic analysis, Biofuels, Bioprod. Bioref. 12 (2018) 56-67, https://doi.org/10.1002/bbb.1828.

[15] M. Brito, F. Martins, Life cycle assessment of butanol production, Fuel 208 (2017) 476-482, https://doi.org/10.1016/j.fuel.2017.07.050.

[16] D. González-Tenorio, I. Valdez-Vazquez, Adaptive laboratory evolution applied to an acidogenic microbial community for enhanced butanol production from corn stover cellulose. Fuel Submitted (2022).

[17] A. Sanchez, G. Magaña, D. Gomez, M. Solís, R. Banares-Alcantara, Bidimensional sustainability analysis of lignocellulosic ethanol production processes. Method and case study. Biofuels, Bioprod. Bioref. 8 (2014) 670-685, https://doi.org/10.1002/bbb.1512.

[18] D. González-Tenorio, K.M. Muñoz-Páez, G. Buitrón, I. Valdez-Vazquez, Fermentation of organic wastes and CO_2 + H_2 off-gas by microbiotas provides short-chain fatty acids and ethanol for n-caproate production, J. CO_2 Util. 42 (2020) 101314, https://doi.org/10.1016/J.JCOU.2020.101314.

[19] H. Kim, O. Choi, B. S. Jeon, W. Choe, B. Sang, Impact of feedstocks and downstream processing technologies on the economics of caproic acid production in fermentation by *Megasphaera elsdenii* T81, Bioresour. Technol. 301 (2020) 122794, https://doi.org/10.1016/j.biortech.2020.122794.

[20] A. C. Dimian, C. S. Bildea, A. A. Kiss, Chapter 13 - Pinch Point Analysis, Editor(s): A. C. Dimian, C. S. Bildea, A. A. Kiss, Comput. Aided Chem. Eng., Elsevier, 35 (2014) 525-564, https://doi.org/10.1016/B978-0-444-62700-1.00013-9.

[21] International Organization for Standardization. ISO 14040- Environmental management- Life cycle assessment- Principles and framework. [Internet]. Available at: 2006. https://www.iso.org/obp/ui#iso:std:iso:14040:ed-2:v1:es. [Accessed 15 July 2021].

[22] A. Bonomi, O. Cavalett, M. Pereira da Cunha, M.A.P. LimaVirtual biorefinery-an optimization Strategy for renewable carbon Valorization. Switzerland:
Springer International Publishing, Cham (2016), 10.1007/978-3-319-26045-7.

[23] W.D. Seider, J.D. Seader, D.R. Lewin, S. Widagdo, Product and Process Design Principles: Synthesis, Analysis and Design, (3rd ed), Wiley, New York, (2009), 728 pp.

[24] Banxico, 2021. Interest rate. Bank of Mexico. (Accessed April 28, 2021)
In Spanish: [Internet]. Available at: https://www.banxico.org.mx/tipcamb/main.do?page=tas&idioma=sp

[25] A. M. Karimi, K. Karimi, Biobutanol production from corn stover in the US, Ind Crops Prod. 129 (2019) 641-653, https://doi.org/10.1016/j.indcrop.2018.12.054.

[26] Alibaba Group, https://www.alibaba.com/ (accessed February 10, 2021), https://spanish.alibaba.com/g/nahco3-manufacturers.html

[27] D.E.R. Silva, J. Bonhivers, R. M. Filho, M. A. Pinto, Biochemical conversion of sugarcane bagasse into the alcohol fuel mixture of isopropanol-butanol-ethanol (IBE): Is it

economically competitive with cellulosic ethanol?, Bioresour. Technol. 314 (2020) 123712, https://doi.org/10.1016/j.biortech.2020.123712.

[28] Alibaba Group, https://www.alibaba.com/ (accessed April 10, 2021), https://spanish.alibaba.com/g/caproic-acid.html

[29] M. Palmeros Parada, P. Osseweijer, J. A. Posada Duque, Sustainable biorefineries, an analysis of practices for incorporating sustainability in biorefinery design, Ind Crops Prod. 106 (2017) 105-123, https://doi.org/10.1016/j.indcrop.2016.08.052.

[30] S&P Global Platts, Latest oil, energy & Metals news, market data and analysis (accessed July 16, 2021)

In Spanish: [Internet]. Available at: https://www.whitehouse.gov/wp-content/uploads/2021/02/TechnicalSupportDocument_SocialCostofCarbonMethaneNitrousOxide.pdf.

[31] CEA, Comisión Estatal de Aguas. Tarifas de agua, https://www.ceaqueretaro.gob.mx (2021) (accessed July 20, 2021)

https://www.ceaqueretaro.gob.mx/wp-content/uploads/2021/03/TARIFAS-METRO-A-METRO-ABRIL-JUNIO-2021-QRO.pdf. [Accessed 20 July 2021].

[32] J. Satyavolu, J. G. D. Tadimeti, R. Thilakaratne, Xylose production and the associated integration for biocoal production,

Energy Convers. Manag. Energy Convers. Manag.: X, 10, (2021) 100073, https://doi.org/10.1016/j.ecmx.2020.100073.

[33] Comisión IMSS-SNT. Tabulador de sueldos vigentes para personal de base, http://www.sntss.org.mx/filesUpdates/tabulador-de-sueldos.pdf (2021) (accessed July 24, 2021).

[34] A. Sanchez, G. Magaña, M.I. Partida, S. Sanchez, Bi-dimensional sustainability analysis of a multi-feed biorefinery design for biofuels co-production from lignocellulosic residues and agro-industrial wastes, Chem. Eng. Res. Des. 107 (2016) 195-217, https://doi.org/10.1016/j.cherd.2015.10.041.

[35] J. Xu, J. Hao, J.J.L. Guzman, C. M. Spirito, L. A. Harroff, L. T. Angenent, Temperature-phased conversion of acid whey waste into medium-chain carboxylic acids via lactic acid: no external e-donor, Joule 2 (2) (2018) 280-295, https://doi.org/10.1016/j.joule.2017.11.008.

[36] J. Chwialkowska, A. Duber, R. Zagrodnik, F. Walkiewicz, M. Łężyk, P. Oleskowicz-Popiel, Caproic acid production from acid whey via open culture fermentation – Evaluation of the role of electron donors and downstream processing, Bioresour. Technol. 279 (2019) 74-83, https://doi.org/10.1016/j.biortech.2019.01.086.

[37] W. de A. Cavalcante, R. C. Leitão, T. A. Gehring, L. T. Angenent, S. T. Santaella, Anaerobic fermentation for n-caproic acid production: A review, Process Biochem, 54 (2017) 106-119. https://doi.org/10.1016/j.procbio.2016.12.024.

[38] Z. Li, Y. Yu, J. Sun, D. Li, Y. Huang, Y. Feng, Effect of Extractives on Digestibility of Cellulose in corn stover with liquid hot water pretreatment. Bioresources. 11 (2016) 54-70, https://doi.org/10.15376/biores.11.1.54-70.

[39] X. Sun, H. K. Atiyeh, R. L. Huhnke, R. S. Tanner, Syngas fermentation process development for production of biofuels and chemicals: A review, Bioresour. Technol. Reports, 7 (2019) 100279, https://doi.org/10.1016/j.biteb.2019.100279.

[40] M. Gao, Y. Tashiro, Q. Wang, K. Sakai, K. Sonomoto, High acetone–butanol–ethanol production in pH-stat co-feeding of acetate and glucose, Journal of Bioscience and Bioengineering, 122 (2) (2016) 176-182, https://doi.org/10.1016/j.jbiosc.2016.01.013.

[41] C. E. Isom, M. A. Nanny, R. S. Tanner, Improved conversion efficiencies for n-fatty acid reduction to primary alcohols by the solventogenic acetogen "*Clostridium ragsdalei*", J. Ind. Microbiol. Biotechnol., 42 (1) (2015) 29–38, https://doi.org/10.1007/s10295-014-1543-z.

[42] R.K. Thauer, K. Jungermann, K. Decker, Energy conservation in chemotrophic anaerobic bacteria, Bacteriological reviews 41 (1) (1977), 100-180. https://doi.org/10.1128/br.41.1.100-180.1977.

[43] J.R. Phillips, R.L. Huhnke, H. K. Atiyeh, syngas fermentation: a microbial conversion process of gaseous substrates to various products, Fermentation 3 (2) (2017) 28, https://doi:10.3390/fermentation3020028.

[44] A. Fernández-Naveira, M. C Veiga., C. Kennes, Selective anaerobic fermentation of syngas into either C2-C6 organic acids or ethanol and higher alcohols, Bioresour. Technol. 280 (2019) 387-395, https://doi.org/10.1016/j.biortech.2019.02.018.

[45] T.I.M. Grootscholten, K.J.J. Steinbusch, H.V.M. Hamelers, C.J.N. Buisman, High rate heptanoate production from propionate and ethanol using chain elongation, Bioresour. Technol. 136 (2013) 715-718, https://doi.org/10.1016/j.biortech.2013.02.085

7.0 Conclusiones y Perspectivas

Por medio de la experimentación de la fermentación acidogénica se tienen cuantificados los productos y los géneros microbianos que participan. Se logra concluir que el consorcio nativo del rastrojo de maíz tuvo la capacidad de trabajar como un bioproceso consolidado al hidrolizar y fermentar los polisacáridos en H_2 y una mezcla de AGV con una composición de 59% de acetato, 26% de butirato, y 15% de propionato. La productividad de H_2 incrementó con el tiempo de operación, y debe profundizarse el estudio de sus causas. Aunado a esto, la actual composición de AGV en la acidogénesis no favorece el rendimiento de butanol en bioprocesos *aguas abajo,* por lo que se requieren otros trabajos enfocados en modificar la composición de AGV donde el ácido butírico sea mayoritario.

Se determinó que la producción de butanol con un corsorcio mirobiano a partir de rastrojos de maíz aumentó 1.5 veces a un pH de 7.5 en comparación con un pH de 6.5.

La producción de butanol con un corsorcio mirobiano a partir de rastrojos de maíz solo fue posible en presencia de ácido butírico como precursor. Por el contrario, mezclas de ácidos grasos volátiles enriquecidas con ácidos acético o propionico inhiben la producción de butanol.

De la evaluación de las estrategias para incrementar la concentración de butanol por un consorcio microbiano (bioaumentación *versus* evolución adaptativa en laboratorio), solo la evolución adaptativa en laboratorio fue eficiente para lograr una concentración de butanol satisfactoria > 10 g/L. Sin embargo, a pesar de estos resultados favorables, el análisis económico indica que los costos de producción aún no son competitivos y deben seguir los estudios para incrementar el rendimiento.

En la fermentación de gases, fue posible controlar el tipo de producto de fermentación obtenido a partir de CO_2/H_2 a través del tipo de inóculo y el medio de cultivo. El acoplamiento de la acidogénesis con la fermentación de gases resultará en mayores rendimientos al incrementar la productividad de H_2, el cual sirve como donador de electrones durante la producción de butanol.

Se estableció un modelo de biorrefinería en cascada para la producción de butanol sustentable a partir de un análisis que emplea la monetización de un índice global que incluye indicadores económicos, ambientales y sociales. El grado de sustentabilidad dependió en gran medida del dominio ambiental, con una contribución importante del indicador de eficiencia energética (EER). Si bien este trabajo fue existoso en proponer un modelo de biorrefinería más sustentable que el modelo base, se identificó que la composición de AGV y productividad de H_2 en la acidogénesis son críticos para favorecer o perjudicar los bioprocesos "*aguas abajo*" de biofijación de CO_2 y fermentación ABE.

Appendix A. Mass balances parameters

Table A1. Reactions (Rxn), Conversion (Conv) and concentrations (Con) for acidogenic fermentation

Rxn	Conv, %	Ref.
1.00 Other solids + 1.0 H_2O→1.00 Glucose	3	Li et al, 2016
1.0 Glucose + 2.0 H_2O → 2.0 Acetate + 2.0 CO_2 + 4.0 H_2	50	González-Tenorio et al., 2020
1.0 Glucose + 2 H_2O → 1.0 Butyrate +2.8 CO_2 + 2.0 H_2	100 %	
1.0 Xylan + 1.0 H_2O + → 1.0 Xylose	69 %	
Rxn	**Con, g/L**	**Ref.**
1.7 H_2O +1.0 Xylose → 1.7 Acetate +1.7 CO_2+3.3 H_2	Acetate 8.5	González-Tenorio et al., 2020
1.0 Xylose → 0.83 Butyrate + 1. 7 CO_2+1. 7 H_2	Butyrate 2.7	
1.0 Xylose → 5.0 Biomass + 1.5 H_2O	Biomass 0.40	

Table A2. Reactions (Rxn), Conversion (Conv) and concentrations (Con) for ABE fermentation reactor.

Rxn	Conv, %	Ref
1.00 Cellulose + 1.0 H_2O → 1.00 Glucose	100 %	González- Tenorio et al., 2021
1.0 Glucose → 6.0 Biomass + 1.8 H_2O	2.0%	
Rxn	**Con, g/L**	**Ref.**
1.0 Glucose + 1.0 H_2O → 1.00 Acetone+ 3.0 CO_2 + 4.0 H_2	Acetone, 6.0	González- Tenorio et al., 2021
1.0 Glucose → 1.0 Butanol +2.00 CO_2 + 1.0 H_2	Butanol, 13.8	
1.0 Glucose → 2.0 Ethanol +2.00 CO_2	Ethanol, 1.3	

Table A3. Reactions (Rxn), Conversion (Conv) and concentrations (Con) for anaerobic digestion reactor.

Rxn	Conv, %	Ref.
1.0 Acetate. → 2.4 Biomass	3	Sánchez et al., 2014
1.0 Butyrate.→ 3.6 Biomass	3	
1.0 Acetate. → 1.0 CO_2+ 1.0 CH_4	95	
1.0 Butyrate + 1.0 H_2O → 1.5 CO_2+ 2.5 CH_4	90	

Table A4. Scenarios evaluated on the biorefinery model with biological CO_2 fixation for butanol production (MCO2ButOH).

Scenario	Rxn	Ref.
MCO_2ButOH-I	12.0 H_2 +4.0 CO_2→1.0 C_4H_9OH + 7.0 H_2O	Sun et al., 2019
MCO_2ButOH -II	12.0 H_2 +4.0 CO_2→1.0 C_4H_9OH + 7.0 H_2O. 1.0 Butyrate + 2.0 H_2 → 1.0 C_4H_9OH + 1.0 H_2O 1.0 Acetate+ 4.0 H_2 → 1.0 C_4H_9OH + 3.0 H_2O	Sun et al., 2019 Gao et al., 2016 Isom et al., 2015 Thauer, 1977

Table A5. Scenarios evaluated on the biorefinery model with biological CO_2 fixation for caproate production (MCO_2Cap)

Scenario	Rxn	Ref.
MCO_2Cap	6 H_2 + 2 CO_2 → C_2H_5OH + 3 H_2O 12 C_2H_5OH + 3 Acetate → 5 Caproate + 4 H_2o + 8.0 H_2O Ethanol + Butyrate → Caproate + H_2O	Phillips et al., 2017 Cavalcante et al., (2017)

Appendix B.

Conditioning (milling)

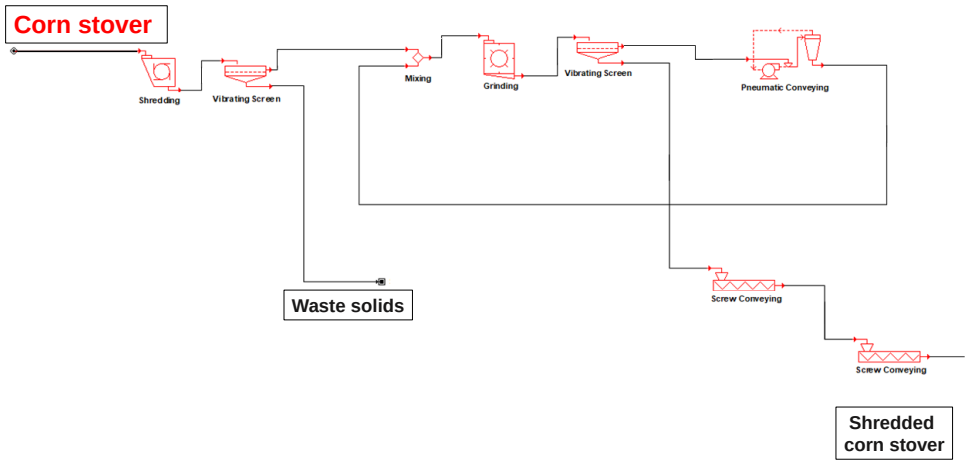

Figure B1. Unit operations diagram of conditioning stage.

Two crushers with a capacity of 20.83 Mton/h and one crusher with a capacity of 23.89 ton/h reduce the particle size to 3 ± mm.

Acidogenic fermentation

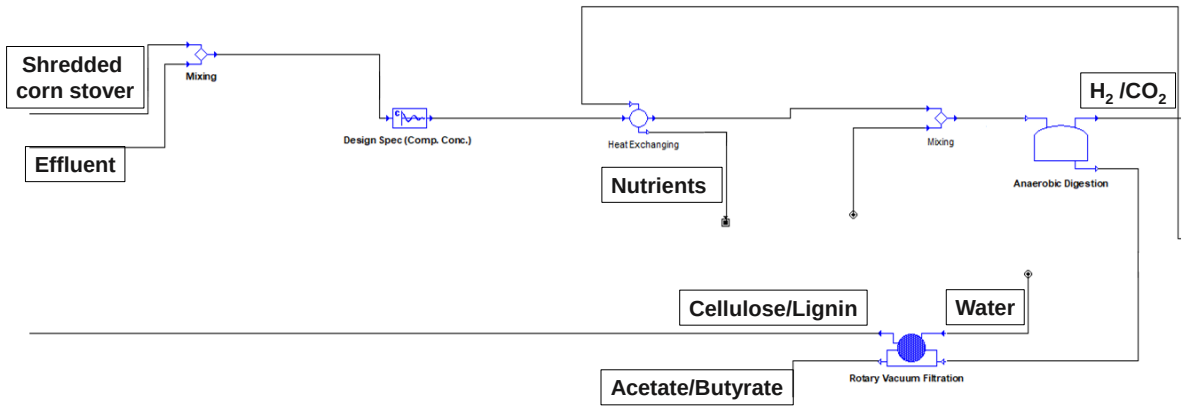

Figure B2. Process diagram of acidogenic fermentation stage.

The acidogenesis reactor operates at a hydraulic retention time (HRT) of 12 h (Valdez-Vazquez & Sanchez, 2018), mesophilic temperature of 37 °C, pH of 6.5, and a total solid content of 10% (w/w) under non-sterile conditions (González-Tenorio et al., 2020a). The acidogenesis reactor operates in continuous mode in a single anaerobic biodigester built-in

concrete of a 5,441 m^3 capacity. The acidogenesis reactor produces 286 kg/h of H_2, 3859 kg/h of CO_2, 3455 kg/h of acetate (concentration of 8.5 g/L), and 1499 kg/h of butyrate (concentration of 3.7 g/L).

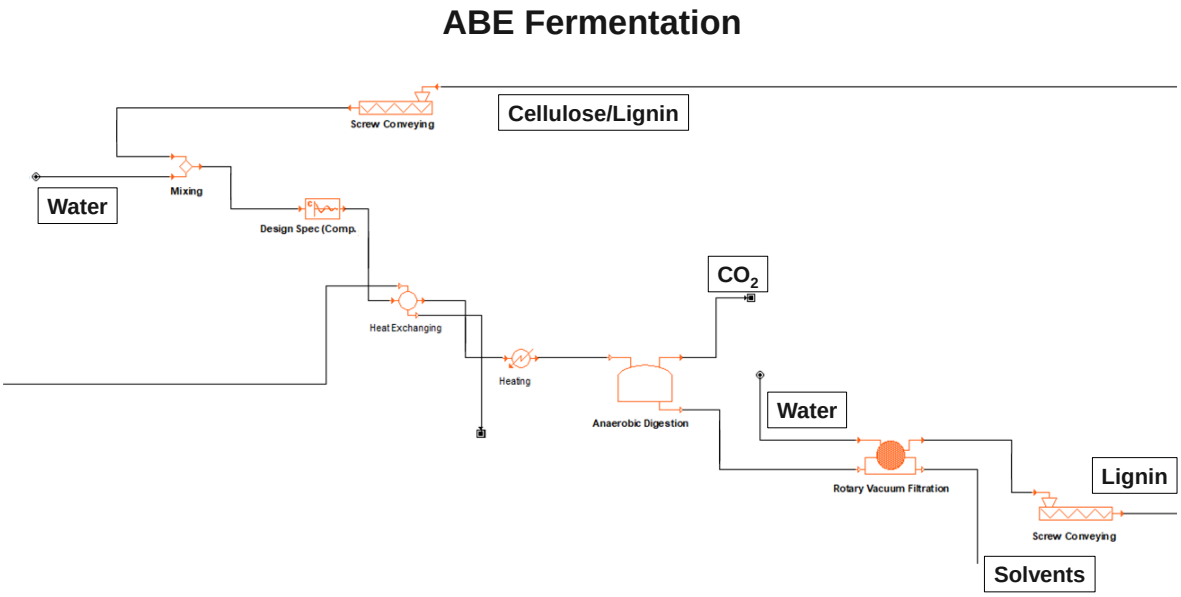

Figure B3. Process diagram of ABE fermentation stage

The ABE fermentation reactor operates at an HRT of 96 h, mesophilic temperature of 37 °C, pH of 7.5, and a total solid content of 10 % (w/w) under non-sterile conditions (González-Tenorio et al., 2021). The ABE fermentation reactor operates in continuous mode in four anaerobic biodigesters built-in concrete of a 3629 m^3 capacity.

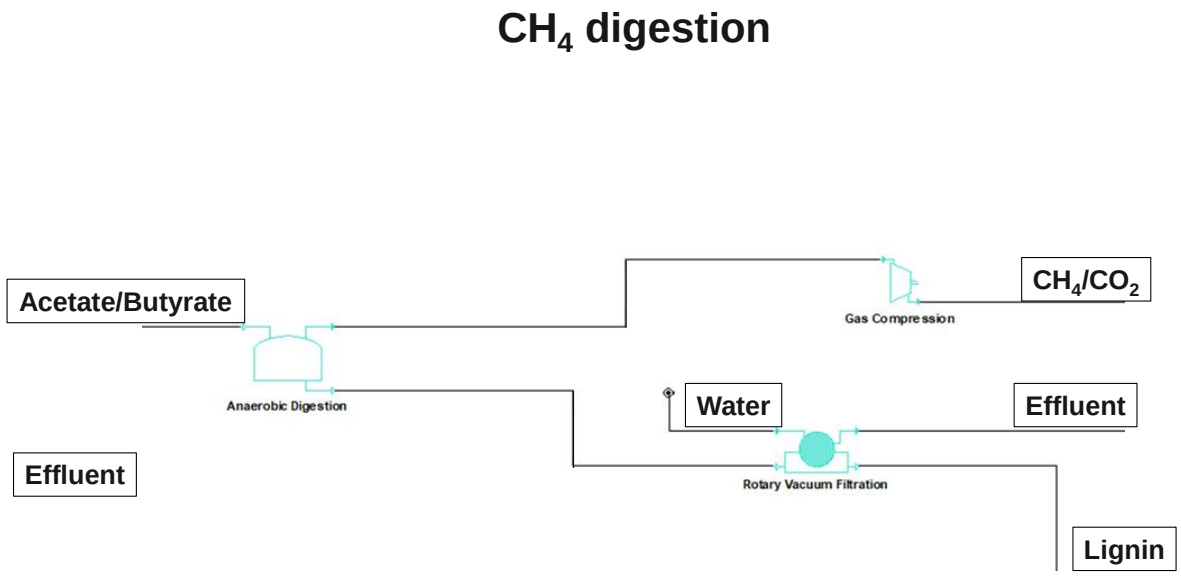

Figure B4. Process diagram of CH_4 digestion stage.

The anaerobic digestion reactor operates at an HRT of 10 h, 37 °C, pH of 7.0 under non-sterile conditions (Valdez-Vazquez & Sanchez, 2018). The anaerobic digestion operates in continuous mode in a single biodigester built-in concrete of a 3,667 m^3 capacity.

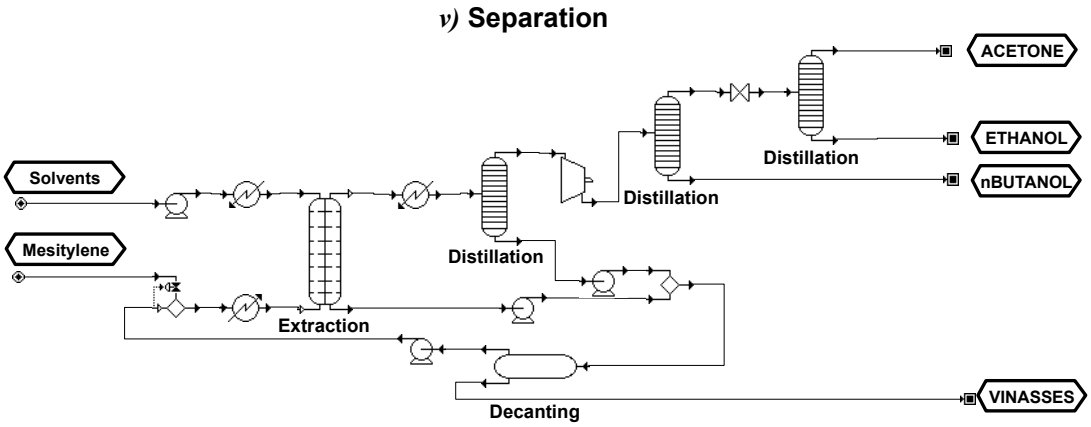

Figure B5. Process diagram of separation stage.

The mass flow represents 243,374 kg/h with a concentration of 13.8 g/L of butanol. A hybrid separation system purifies butanol consisting of two treatments: the first treatment uses an extractive column using mesitylene as an extractive agent and the second treatment consists of three distillation columns at pressures of 1.5, 2.0, 0.5 bar according to the previously reported configuration (Sánchez et al., 2017; Valdez-Vazquez & Sánchez, 2018).

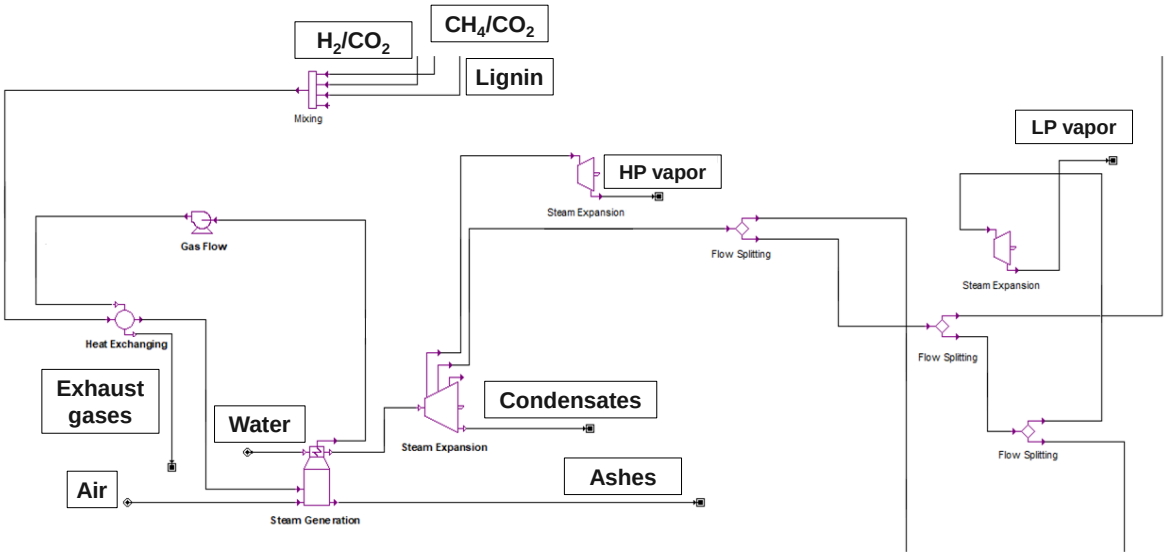

Figure B6. Process diagram of cogeneration stage.

The steam generator operates at 86 bar and 510 °C, the steam powers an expansion turbine generating steam at low and high pressure (Valdez-Vazquez & Sanchez, 2018).

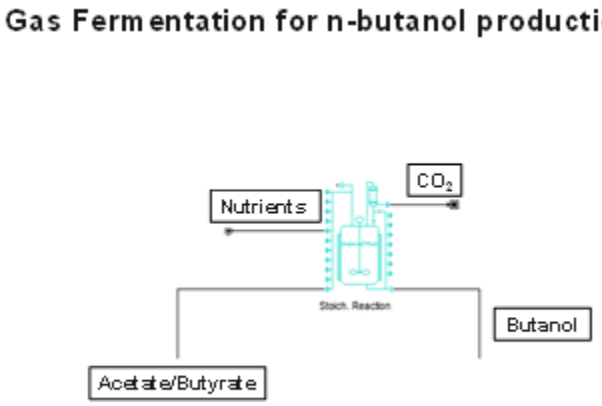

Figure B7a. Process diagram of gas fermentation stage for butanol production.

The gas fermentation reactor operates at an HRT of 2 h, mesophilic temperature of 37 °C (Fernández-Naveira et al., 2019), pH of 6.5, under non-sterile conditions with mineral medium (González-Tenorio et al., 2020a). The gas fermentation reactor operates in continuous mode in one anaerobic digesters built-in stainless steel of a 34.5 m^3 capacity.

Gas Fermentation for n-butanol production

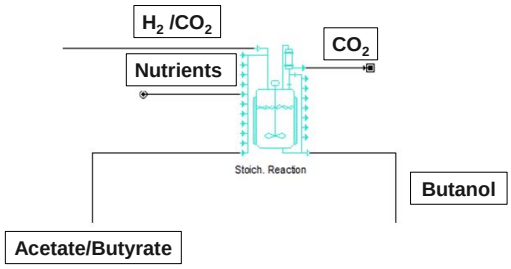

Figure B7b. Process diagram of gas fermentation stage for butanol.

The gas fermentation reactor operates at an HRT of 2 h, mesophilic temperature of 37 °C (Fernández-Naveira et al., 2019), pH of 6.5, under non-sterile conditions with mineral media (González-Tenorio et al., 2020a). The gas fermentation reactor operates in continuous mode in eighteen anaerobic digesters built-in stainless steel of a 34.5 m^3 capacity.

Gas Fermentation for n-caproate production

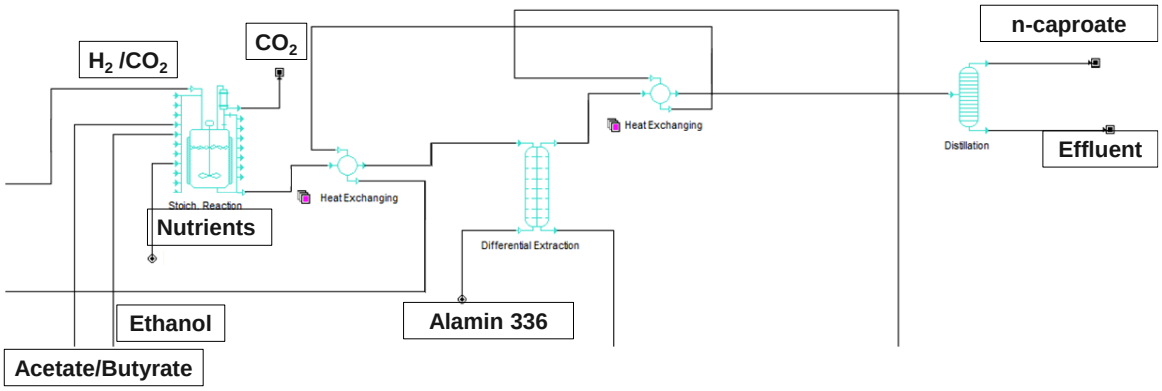

Figure B8. Process diagram of gas fermentation stage for caproate production.

The gas fermentation reactor operates at an HRT of 4h, mesophilic temperature at 37 °C (Grootscholten et al., 2013), pH of 6.5, under non-sterile conditions with 4 g/L of urea and 2 g/L of KH_2PO_4 as nutrients (González-Tenorio et al., 2020b). The gas fermentation reactor operates in continuous mode in five anaerobic digesters anaerobic digester built-in stainless steel of a 36 m^3 capacity.

Appendix C.

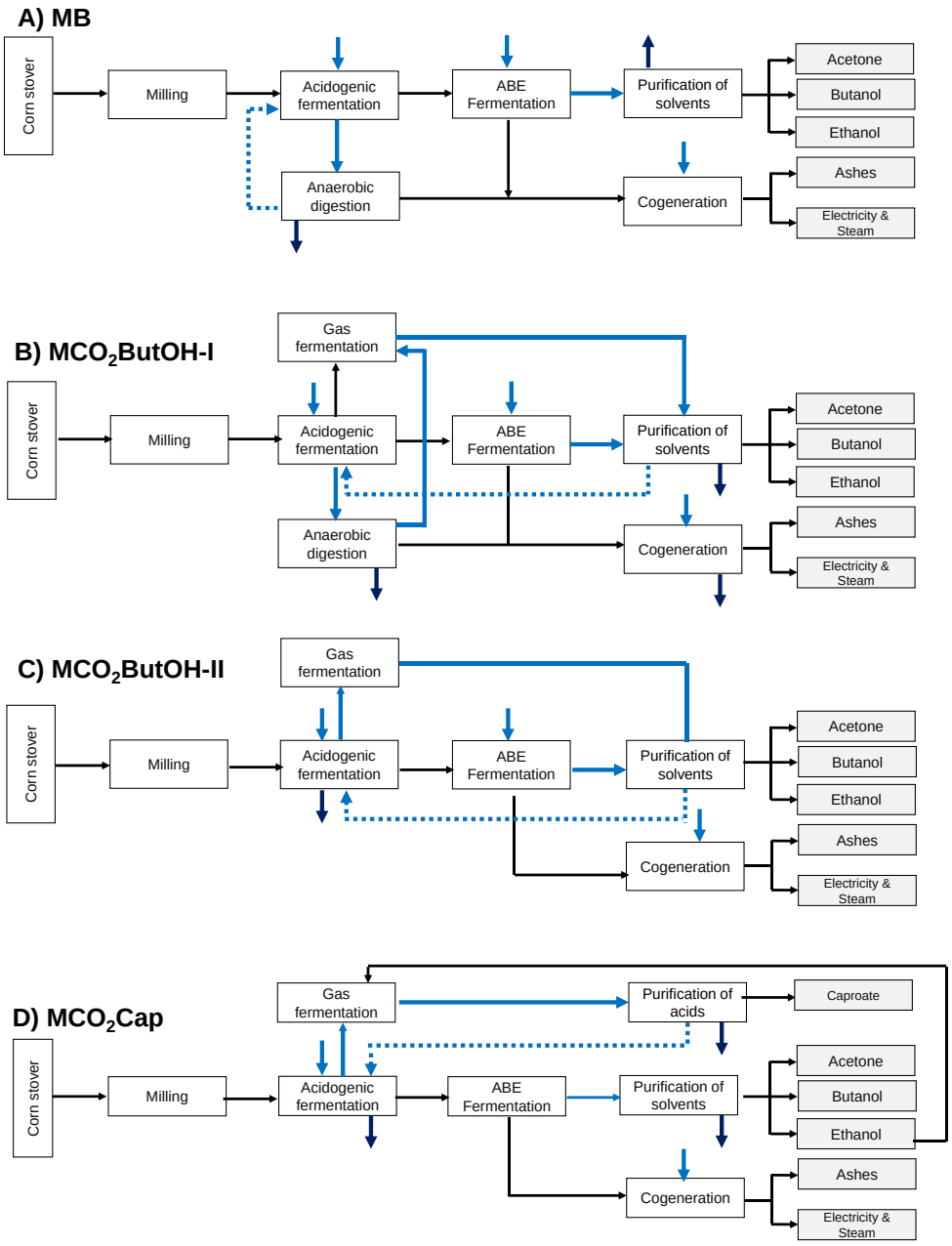

Figure C1. Schematic representation of the direction of water currents, water (↓), wastewater (↓) and water recirculation (◄┈┈).

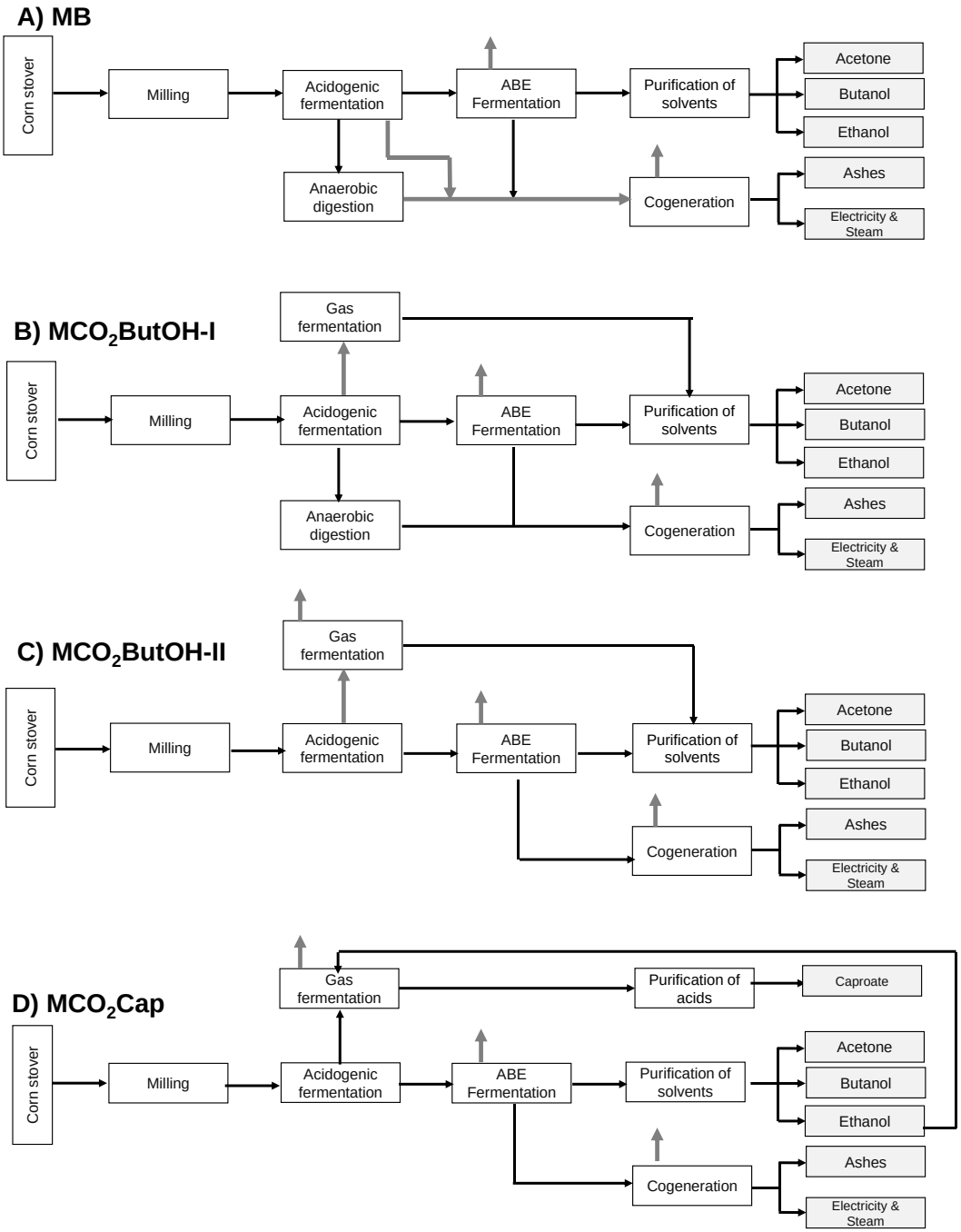

Figure C2. Schematic representation of the direction of CO_2 currents, CO_2 (→) and Emissions of CO_2 (↑).