UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS QUIMICAS

TESIS DOCTORAL

Producción de ácido succínico con Actinobacillus succinogenes 130Z

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Itziar Arias Escanciano

Directores

Victoria E. Santos Mazorra
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FACULTAD DE CIENCIAS QUÍMICAS

PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA

PRODUCCIÓN DE ÁCIDO SUCCÍNICO CON *ACTINOBACILLUS SUCCINOGENES* 130Z

Presentada por:

Itziar Arias Escanciano

para optar al título de Doctor por la Universidad Complutense de Madrid

Directores:

Victoria E. Santos Mazorra
Miguel Ladero Galán

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MADRID

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CERTIFICAN: Que la presente memoria titulada "*PRODUCCIÓN DE ÁCIDO SUCCÍNICO CON ACTINOBACILLUS SUCCINOGENES 130Z*" constituye la memoria que presenta Itziar Arias Escanciano para optar al grado de doctor, y que ha sido realizada mayoritariamente en los laboratorios del Departamento de Ingeniería Química y de Materiales de la Universidad Complutense de Madrid bajo su dirección

Y para que así conste, firman el presente certificado en Madrid a 23 de MARZO de 2023

Fdo.: Victoria E. Santos Mazorra

Fdo.: Miguel Ladero Galán

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RESUMEN

El ácido succínico o ácido butanodioico es un compuesto con multitud de aplicaciones en la industria farmacéutica y alimentaria, así como para la producción de resinas, pigmentos, recubrimientos y con gran potencial para la generación de plásticos biodegradables. Este ácido es considerado clave en el marco de las biorrefinerías y la bioeconomía, siendo considerado uno de los 12 principales productos químicos de plataforma según el Departamento de Energía de los Estados Unidos (*United States Department Of Energy* - US DOE).

Tradicionalmente, la producción de ácido succínico se ha realizado a partir de recursos fósiles, principalmente, mediante procesos de hidrogenación catalítica u oxidación parafínica. Sin embargo, en las últimas décadas, debido a la creciente preocupación por las consecuencias del cambio climático y la escasez de recursos fósiles, los esfuerzos de investigación se han centrado en la generación de este ácido mediante métodos biológicos basados en procesos de fermentación, intentando aprovechar biomasa residual de diversa índole con el objetivo de desarrollar una economía circular en el contexto de las biorrefinerías. La confluencia del incremento de los costes de la materia prima fósil y del creciente desarrollo de las operaciones biológicas han posibilitado la competitividad económica de estos bioprocesos en el mercado. Sin embargo, todavía hay un gran margen de mejora a la hora de incrementar sus rendimientos y productividades, así como reducir sus costes de operación.

En este contexto, en la presente Tesis se profundiza en el estudio del proceso de producción de ácido succínico mediante la acción de *Actinobacillus succinogenes* 130Z, una bacteria anaeróbica facultativa aislada del rumen del ganado. El objetivo de este trabajo es la optimización de este proceso, estudiar su cinética y fenomenología y aprovechar residuos alimentarios como sustitutos de las fuentes de carbono y de nitrógeno comerciales.

En primer lugar, se estudia la influencia en la producción a nivel de reactor de la realización de sucesivas etapas de adaptación del microorganismo a la fuente de carbono en los crecimientos previos llevados a cabo en botella, así como la realización de dos etapas de inóculo, observándose que el rendimiento de la reacción se incrementa en un 173 % y la productividad en un 227 %.

Posteriormente, se busca la optimización de la producción empleando la operación tipo *batch* mediante la realización de experimentos estudiando distintas condiciones de operación y variables de proceso: concentración inicial de glucosa, concentración inicial de biomasa, concentración de extracto de levadura, velocidad de agitación y caudal de CO_2. Gracias a estos ensayos se puede determinar que a una concentración inicial de biomasa de 0,075 g L^{-1} se obtienen los valores máximos de rendimiento y productividad. Al trabajar con una concentración de 7,5 g L^{-1} de extracto de levadura se genera mayor cantidad de ácido succínico, pero utilizando concentraciones menores, se incrementa la selectividad y a 10 g L^{-1} la velocidad de producción resulta superior. El empleo de concentraciones bajas de la fuente de carbono conduce a mayores concentraciones finales de succínico a expensas de una pérdida de selectividad. En cuanto a la agitación, para lograr una adecuada transferencia gas-líquido, ésta debe estar comprendida entre 200 rpm y 300 rpm, valor a partir del cual se observa estrés hidrodinámico. Los caudales de CO_2 estudiados, no generan diferencias en los resultados, por lo que se concluye que se suministra en exceso.

También se comprueba la viabilidad de la producción de ácido succínico con células en estado de *resting*, es decir, con células metabólicamente activas pero cuyo crecimiento ha sido inhibido por la ausencia de una fuente de nitrógeno en el medio de cultivo. Se infiere la necesidad de realizar etapas previas de adaptación del microorganismo a la fuente de carbono para obtener cantidades de ácido succínico aceptables. Además, la adición de una etapa de preinóculo permite mejorar el desempeño de la reacción. Tras la realización de experimentos a partir de células inmovilizadas en el *biofilm* o en suspensión en el caso de cultivo a distintos tiempos de crecimiento, se determina que los mejores resultados se alcanzan con células libres tras 15 h de crecimiento, logrando producir 8,51 g L^{-1} con un rendimiento del 43 % en una operación tipo *batch* a partir de xilosa. Una vez establecidas estas condiciones, con el objetivo de abaratar costes del proceso, se llevan a cabo producciones en *batch* con *resting cells* cuyo crecimiento había tenido lugar en un medio de cultivo más económico que el que se había empleado hasta ese momento, incrementando, además, el rendimiento en un 23 % y la productividad en un 67 %.

Por otro lado, con el objetivo de optimizar el proceso, se comparan las formas de operación *fed-batch* y *repeated batch*, tanto con células en estado de crecimiento como en estado de *resting*. Al llevar a cabo fermentaciones con células en estado de

crecimiento, se aprecia que las operaciones tipo *repeated batch* son las que permiten alcanzar mejores rendimientos (0,51 g g^{-1}), mientras que en *fed-batch* el rendimiento se reduce en un 31 %. Con *resting cells*, las sucesivas centrifugaciones de las producciones tipo *repeated batch* suponen un estrés excesivo para las células, pero al operar en *fed-batch*, se logran rendimientos del 53 % y selectividades un 36 % superiores que en la operación equivalente realizada con células en crecimiento.

Para situar esta Tesis Doctoral en el contexto de una economía circular, se lleva a cabo el tratamiento de residuos de patata de una fábrica de snacks y residuos de bagazo de cerveza y levadura de cerveza gastada de una cervecería local utilizando ácidos o enzimas. La levadura de cerveza gastada, sometida a la acción de proteasas para obtener un licor de elevado valor proteico, se emplea en sustitución del extracto de levadura comercial, como fuente de carbono. La hidrólisis enzimática del bagazo de cerveza se lleva a cabo en varias etapas en presencia de amilasas, xilanasas, glucanasas, endoproteasas y glucoamilasas, obteniendo un hidrolizado con un porcentaje de xilosa del 84 %. Los residuos de patata se someten, sin embargo, a un tratamiento ácido con el objetivo de generar un hidrolizado rico en glucosa. La posibilidad de emplear estos residuos en fermentaciones tipo *batch* y células en crecimiento queda validada. A partir de los residuos de patata se logra incrementar el rendimiento del proceso un 37 % en relación con su operación equivalente con glucosa pura. Empleando los residuos procedentes de la cervecera simultáneamente como fuente de carbono y de nitrógeno, se logra incrementar el rendimiento en un 45 % y la selectividad en un 52 % respecto al experimento de referencia realizado a partir de xilosa pura y extracto de levadura. Dado que se observó con azúcares puros que, con células en crecimiento, el mejor modo de operación era *repeated batch*, se lleva a cabo experimentos con este tipo de operación a partir de hidrolizado de residuos de patata como fuente de carbono e hidrolizado de levadura de cerveza gastada como fuente de nitrógeno, obteniendo rendimientos del 84 % y selectividades del 79 %.

Por otro lado, para el estudio de la influencia del aporte del CO_2 en el proceso de fermentación, se llevan a cabo fermentaciones en *batch* cortando el suministro de este gas al inicio del proceso y con insuflación continua de este gas. Tras no observarse diferencias en los resultados obtenidos, se determina que la transferencia de CO_2 en el sistema no es el fenómeno limitante de la velocidad global del proceso. Con esta premisa se plantean modelos cinéticos no estructurados – no segregados que se ajustan con precisión a los

datos de evolución temporal del sustrato, productos, subproductos y biomasa en las fermentaciones llevadas a cabo tanto con células en crecimiento como en *resting*, utilizando distintos modos y condiciones de operación e incluso empleando hidrolizados en vez de compuestos comerciales. A pesar del reducido número de parámetros de los modelos, en todas las estimaciones se obtienen ajustes de gran bondad respaldados por los valores de los parámetros estadísticos relevantes.

ABSTRACT

Succinic acid or butanedioic acid is a compound with many applications in the pharmaceutical and food industry, as well as for the production of resins, pigments, coatings and with great potential for the generation of biodegradable plastics. This acid is considered key in the framework of biorefineries and the bioeconomy, being one of the 12 main platform chemicals according to the United States Department of Energy (US DOE).

Traditionally, the production of succinic acid has been carried out from fossil resources, mainly through processes of catalytic hydrogenation or paraffin oxidation. However, in recent decades, due to growing concern about the consequences of climate change and the scarcity of fossil resources, research efforts have focused on the generation of this acid through biological methods based on fermentation processes, trying to take advantage of several kinds of residual biomass with the aim of developing a circular economy in the context of biorefineries. The confluence of the increase in the costs of the fossil raw material and the growing development of biological operations have enabled the economic competitiveness of these bioprocesses in the market. However, there is still a great margin for improvement when it comes to increasing their yields and productivities, as well as reducing their operating costs.

In this context, this Thesis intends to deepen the study of the production process of succinic acid through the action of *Actinobacillus succinogenes* 130Z, a facultative anaerobic bacterium isolated from the rumen of cattle. The objective of this work is to optimize this process, study its kinetics and phenomenology and use food waste as substitutes for commercial carbon and nitrogen sources.

In the first place, the influence on the production at the reactor level of the successive stages of adaptation of the microorganism to the carbon source in the previous growths carried out in the bottle is studied, as well as the performance of two stages of inoculum, observing that the yield of the reaction is increased by 173 % and the productivity by 227 %.

Subsequently, the optimization of production is sought using the batch type operation by carrying out experiments studying different operating conditions and process variables: initial glucose concentration, initial biomass concentration, yeast extract

concentration, agitation speed and flow rate. of CO_2. Thanks to these tests it can be determined that at an initial biomass concentration of 0.075 g L^{-1} the maximum yield and productivity values are obtained. When working with a concentration of 7.5 g L^{-1} of yeast extract, a greater amount of succinic acid is generated, but using lower concentrations, selectivity increases and at 10 g L^{-1} the production rate is higher. The use of low concentrations of the carbon source leads to higher final concentrations of succinic at the expense of a loss of selectivity. Regarding agitation, to achieve an adequate gas-liquid transfer, it must be between 200 rpm and 300 rpm, value from which hydrodynamic stress is observed. The CO_2 flows studied do not generate differences in the results, so it is concluded that it is supplied in excess.

The viability of succinic acid production with cells in a resting state is also checked, that is, with metabolically active cells but whose growth has been inhibited by the absence of a nitrogen source in the culture medium. It is inferred the need to carry out previous stages of adaptation of the microorganism to the carbon source to obtain acceptable amounts of succinic acid. In addition, the addition of a pre-inoculum step allows to improve the performance of the reaction. After carrying out experiments using cells immobilized in the biofilm or in suspension in the case of culture at different growth times, it is determined that the best results are achieved with free cells after 15 h of growth, managing to produce 8.51 g L^{-1} with a yield of 43 % in a batch type operation from xylose. Once these conditions are established, with the aim of lowering the costs of the process, batch productions are carried out with resting cells whose growth had taken place in a more economical culture medium than the one used up to that moment, also increasing performance by 23 % and productivity by 67 %.

On the other hand, with the aim of optimizing the process, the fed-batch and repeated batch modes of operation are compared, both with cells in the growth state and in the resting state. When carrying out fermentations with cells in a state of growth, it can be seen that repeated batch operations are the ones that allow the best yields to be achieved (0.51 g g^{-1}), while in fed-batch the yield is reduced by 31 %. With resting cells, the successive centrifugations of the repeated batch productions suppose excessive stress for the cells but, when operating in fed-batch, yields of 53 % and selectivities 36 % higher than in the equivalent operation carried out with growing cells.

To situate this PhD Thesis in the context of a circular economy, the treatment of potato waste from a snack factory and waste beer bagasse and spent brewer's yeast from

a local brewery is carried out using acids or enzymes. Spent brewer's yeast, subjected to the action of proteases to obtain a liquor with a high protein value, is used instead of commercial yeast extract, as a carbon source. The enzymatic hydrolysis of beer bagasse is carried out in several stages in the presence of amylases, xylanases, glucanases, endoproteases and glucoamylases, obtaining a hydrolysate with a xylose percentage of 84 %. The potato residues are, however, subjected to an acid treatment in order to generate a glucose-rich hydrolysate. The possibility of using these residues in batch type fermentations and growing cells is validated. From potato residues, it is possible to increase the yield of the process by 37 % in relation to its equivalent operation with pure glucose. The residues from the brewery have been used simultaneously as a carbon and nitrogen source, managing to increase the yield by 45 % and the selectivity by 52 % compared to the reference experiment carried out from pure xylose and yeast extract. Given that it had previously been observed that, with growing cells, the best operational mode was repeated batch, it was decided to carry out experiments with this type of operation using hydrolysed potato residues as a carbon source and brewer's yeast hydrolysate. spent as a source of nitrogen, obtaining yields of 84 % and selectivities of 79 %.

On the other hand, to study the influence of the CO_2 contribution in the fermentation process, batch fermentations are carried out, cutting off the supply of this gas at the beginning of the process and with continuous insufflation of this gas. After not observing differences in the results obtained, it is determined that the transfer of CO_2 in the system is not the limiting phenomenon of the global speed of the process. With this premise, unstructured - non-segregated kinetic models are proposed that fit precisely the data on the temporal evolution of the substrate, products, by-products and biomass in the fermentations carried out both with growing and resting cells, using different modes, operating conditions and even using hydrolysates instead of commercial compounds. Despite the small number of parameters of the models, in all the estimations high goodness fits are obtained, supported by the values of the relevant statistical parameters.

1. INTRODUCCIÓN

1 INTRODUCCIÓN

La elevada tasa de crecimiento de la población mundial supone un gran desafío para el cumplimiento de los Objetivos de Desarrollo Sostenible (ODS) [1] , derivando en una gran incertidumbre debida al agotamiento de diversos recursos fósiles y a la escasez o mal reparto de recursos básicos como alimentos, energía y agua potable, ligados, por otra parte, entre sí. En buena parte, esta incertidumbre deriva de problemas ambientales ocasionados por a la contaminación del aire, de los acuíferos y del suelo, que también se refleja en el calentamiento global que experimenta nuestro planeta [2].

Actualmente, la energía generada por combustibles fósiles abastece aproximadamente el 88 % de la demanda mundial, siendo este un requerimiento que probablemente aumente en un 26 % durante los próximos 20 años [3]. Esta tendencia queda patente en la previsión de la demanda de petróleo de los próximos años, pronosticándose que se incremente desde 99,4 barriles/día en 2022 hasta los 104,1 barriles/día en 2026 [4], siendo la región Asia Pacífico donde se ha generado 90% del aumento de este consumo [3].

Sin embargo, el agotamiento de los recursos fósiles no es la única consecuencia de la explotación intensiva de los mismos, siendo también la emisión de gases de efecto invernadero una secuela crítica desencadenante del calentamiento global. Según el Panel Intergubernamental sobre el Cambio Climático (*Intergovernmental Panel on Climate Change* - IPCC), este fenómeno se define como *el aumento de las temperaturas combinadas de la superficie terrestre, el aire y la superficie del mar promediadas durante un período de 30 años* [5,6]. A pesar de los crecientes esfuerzos de las últimas décadas y de los avances realizados por la Convención Marco de las Naciones Unidas sobre el Cambio Climático (CMNUCC), las emisiones de efecto invernadero han continuado incrementando la temperatura promedio de la superficie terrestre 1,1 °C desde la época preindustrial [5]. Se estima que, en el peor de los casos, la trayectoria actual conduzca a un calentamiento de hasta 3,9 ºC para el año 2100. En el caso de que se implementaran todas las medidas determinadas para 2030 entonces se esperaría un incremento de 2,4 ºC y, en el caso de cumplir con todos los compromisos y objetivos a largo plazo, esta cifra se podría reducir hasta los 2,1 ºC. Sin embargo, habría que remontarse hasta épocas

anteriores a la del Pleistoceno para encontrar evidencias de valores de una temperatura terrestre 2 ºC superior a la correspondiente a los niveles preindustriales [7].

Por este motivo, se precisa una alternativa capaz reducir la dependencia global de los combustibles fósiles. El desarrollo de tecnologías para la valorización de la biomasa resulta ser una de las propuestas más robustas, ya que supone una fuente económica y sostenible tanto de energía como de productos químicos y materiales, además de alimentos adicionales tanto para los animales domésticos como para la Humanidad [8].

1.1 LA BIORREFINERÍA, PILAR DE LA BIOECONOMÍA

Según la Organización para la Agricultura y la Alimentación (*Food and Agriculture Organization* - FAO), la bioeconomía está basada en el uso sostenible y circular de recursos y procesos biológicos para producir alimentos, piensos, productos y servicios de base biológica, con un gran potencial sin explotar para favorecer tanto la mitigación como la adaptación y resiliencia al cambio climático [8].

Este modelo económico permite reducir las emisiones procedentes del sistema agroalimentario, responsable de un tercio del total de ellas, reemplazando recursos fósiles y sus correspondientes procesos industriales por materias primas y tecnologías biológicas. En la Figura 1.1 se muestra un esquema en el que se representan varias opciones prometedoras para mitigar las emisiones de Gases de Efecto Invernadero (GEI) desde la perspectiva de la bioeconomía. Estas medidas fueron propuestas en el resumen ejecutivo de la contribución del Grupo de Trabajo III (GTIII) al Sexto Informe de Evaluación (AR6) del IPCC [9].

La bioeconomía abarca todos los sectores y sistemas que dependen de los recursos biológicos (animales, plantas, microorganismos y biomasa derivada, incluidos los desechos orgánicos), sus funciones y principios. Incluye y vincula: los ecosistemas terrestres y marinos y los servicios que brindan; sectores de producción primaria que utilizan y producen recursos biológicos (producción agrícola y ganadera, silvicultura, pesca y acuicultura), trayendo nuevas oportunidades comerciales, inversión y empleo; y todos los sectores que utilizan recursos y procesos biológicos para producir alimentos, piensos, bioproductos, energía y servicios (industria química y del plástico, construcción,

industria farmacéutica, industria textil, gestión de residuos y biotecnología), fomentando el desarrollo regional y apoyando a las empresas de pequeña-mediana escala [10–14].

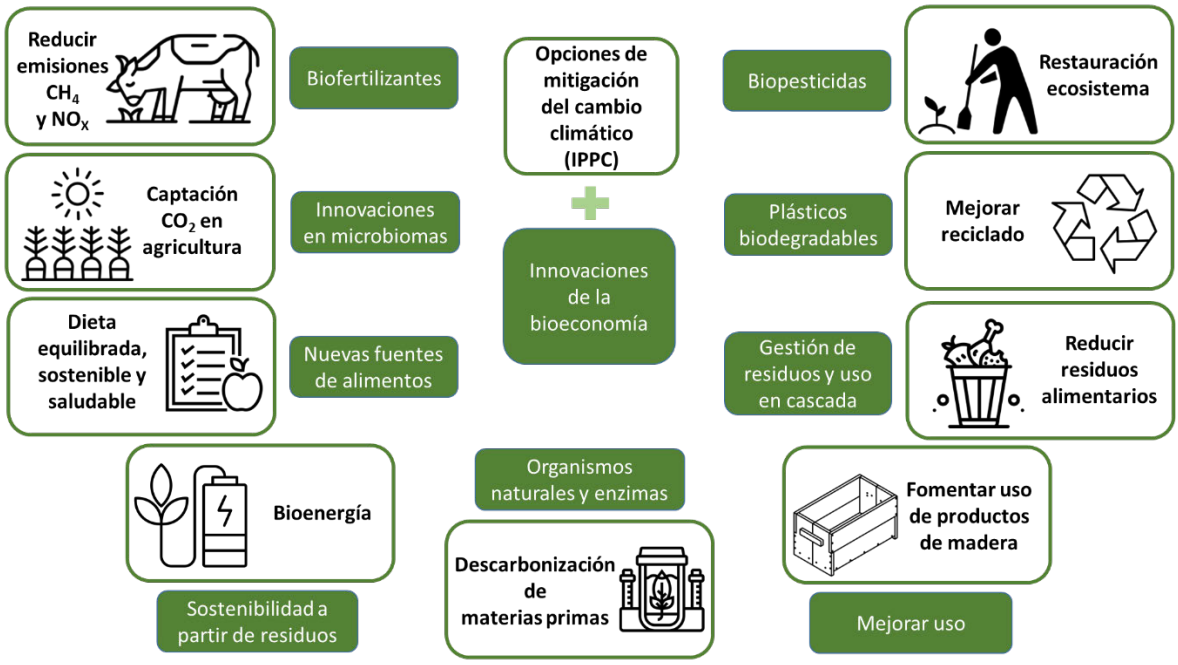

Figura 1.1. Opciones de mitigación del cambio climático propuestas por el IPPC y sus correspondientes medidas innovadoras a partir de la bioeconomía.

Para poder materializar este modelo económico resulta imprescindible la implantación de instalaciones sostenibles que permitan generar bioenergía y bioproductos a partir de diversas materias primas de biomasa mediante la incorporación de tecnologías de conversión de base biológica, en otras palabras, el desarrollo de biorrefinerías. Según el Laboratorio de Energías Renovables Nacional Americano (*National Renewable Energy Laboratory* – NREL), una biorrefinería es *una instalación que integra procesos y equipos de conversión de biomasa para producir combustibles, energía y productos químicos a partir de biomasa*. Desde este centro se considera que las biorrefinerías son la vía más prometedora para la creación de una nueva industria doméstica de base biológica [15].

1.1.1 Tipos y química de la biomasa

La biomasa es cualquier materia orgánica que esté disponible de forma renovable o recurrente, incluidos los árboles y cultivos energéticos, los residuos de cultivos de alimentos y piensos agrícolas, las plantas acuáticas, la madera y los residuos de madera,

los desechos animales y otros materiales de desecho [16]. A continuación, se describen los cuatro tipos de biomasa más empleados.

-Cultivos energéticos. Son cultivos de rápido crecimiento en plantaciones de elevada densidad, de manera extensiva, con el objetivo de suministrar elevadas cantidades de biomasa de calidad constante para las biorrefinerías. Principalmente, son cultivos herbáceos (plantas perennes de cosecha anual como el bambú, sorgo dulce, miscanthus, pasto de trigo, alpiste, heno de alfalfa, entre otros), leñosos (árboles de madera dura y crecimiento rápido, cosechados que se cosechan a los 5-8 años de la plantación, como el álamo híbrido, el sauce híbrido, el arce plateado, el álamo, el fresno verde, el nogal negro o el sicomoro,), agrícolas (cultivos oleaginosos, cereales, cultivos azucareros y ricos en fécula) y acuáticos (algas, algas marinas gigantes, otras algas marinas, microflora marina..) [16,17].

- Residuos y desechos agrícolas. Principalmente son tallos y hojas que no tienen salida comercial, por ejemplo, el bagazo de caña de azúcar o sorgo dulce, paja de trigo, arroz y cebada, cascarilla de arroz y nuez, rastrojo de maíz o el hueso de aceituna. Uno de los principales beneficios del empleo de estos residuos es la eliminación de la necesidad de sacrificar tierras cultivables para su obtención. Dentro de esta categoría, también se considera el estiércol animal (de bovinos, pollos y cerdos) y aquellos subproductos y desechos del procesamiento de la biomasa, como el aserrín, corteza, ramas y hojas/agujas sin usar que se producen durante el procesamiento de la madera para bioproductos o pulpa [16,18,19].

-Desechos y residuos forestales. Biomasa no cosechada en los sitios de tala así como aquella resultante de las operaciones de gestión forestal (raleo de rodales jóvenes y eliminación de árboles muertos y moribundos). Es preferible que su aprovechamiento tenga lugar cerca de su fuente para evitar los gastos asociados al transporte, lo que supone una considerable limitación [16,19].

-Residuos industriales y municipales. En esta categoría se incluyen los residuos sólidos municipales (desechos de papel, cartón, desechos de jardín), los lodos de depuradora y los residuos industriales. Cabe destacar que los residuos alimentarios

industriales y posconsumo residenciales, comerciales e institucionales suponen una de las fuentes de biomasa con mayor potencial y versatilidad [16,18].

1.1.2 La actividad de una biorrefinería

La dinámica de una biorrefinería se puede considerar un sistema análogo, en ciertos aspectos, al de las refinerías de petróleo. En la Figura 1.2 se muestra un esquema de los tipos de procesos de transformación de la biomasa que se encuentran en una instalación de biorrefinado [20].

El refinado de base biológica se inicia con el fraccionamiento de los componentes de la biomasa (celulosa, almidón, azúcares, lignina, aceites, biogás, fracciones proteicas, metabolitos vegetales y microbiano) mediante procesos primarios, es decir, operaciones de acondicionamiento, descomposición y pretratamiento de la biomasa. Los productos obtenidos son denominados plataformas de biorrefinería (plataforma de azúcares, plataforma de aceites…), los cuales se someten a procesos de conversión y procesado adicionales que generan una amplia variedad de productos. Estos pueden ser procesados total o parcialmente en precursores, que terminan siendo refinados total o parcialmente en sucesivas conversiones [21].

En la plataforma de gas de síntesis la biomasa sufre una oxidación parcial (aire, vapor u oxígeno) a 600 - 900ºC, dando lugar principalmente a CO, CO_2, N_2, CH_4, H_2 Y H_2O en proporciones variables. El gas de síntesis ($CO + H_2$) limpio se puede emplear para generar energía, alcoholes, combustibles (síntesis de Fischer-Tropsch) o puede ser conducido a procesos de fermentación con el objetivo de obtener metanol, etanol, amoniaco o ácidos carboxílicos entre otros compuestos [22–24].

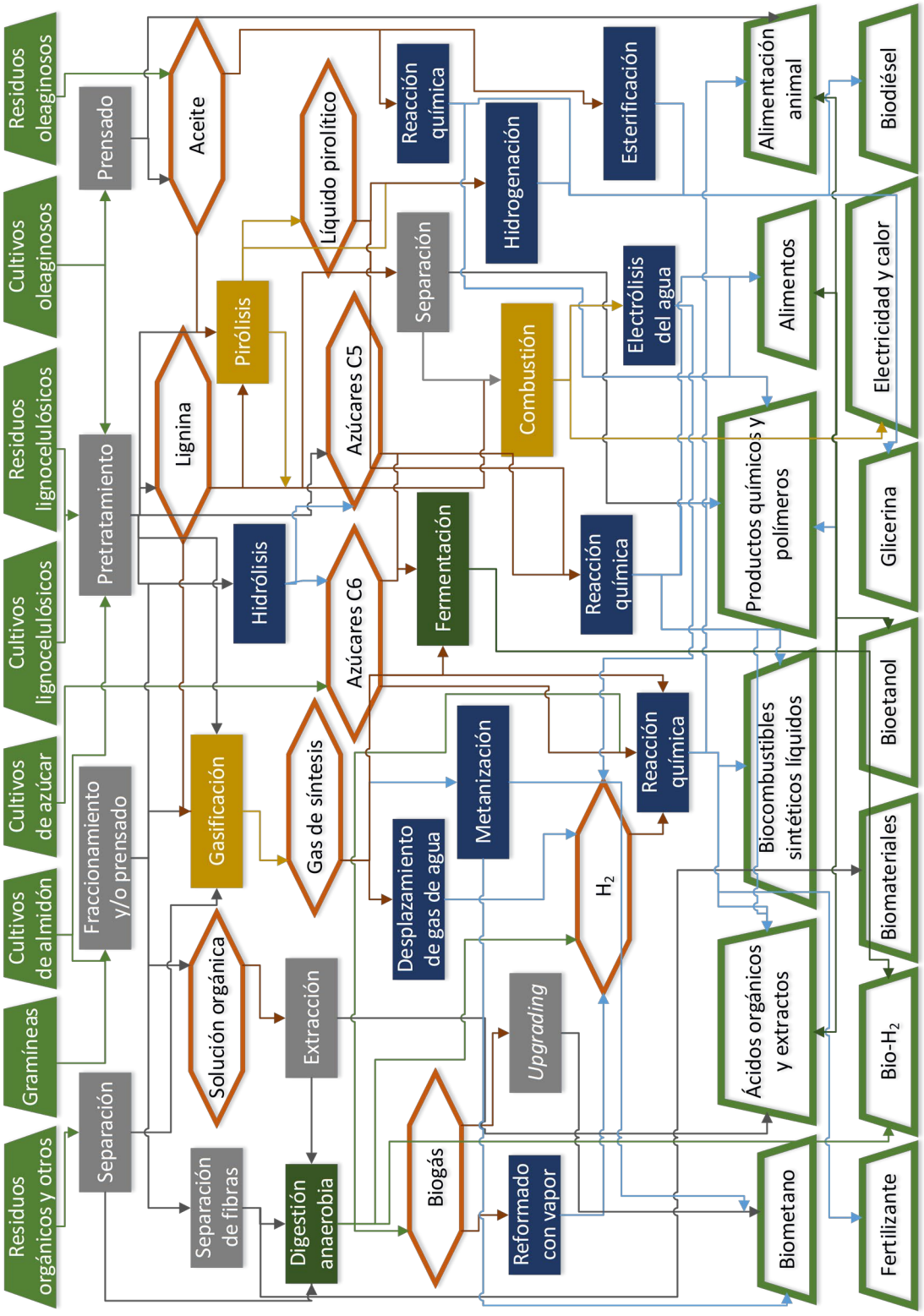

Figura 1.2. Principales tipos de procesos de conversión de la biomasa en una biorrefinería

La plataforma de aceite de pirólisis somete a la biomasa, en ausencia de oxígeno para evitar la combustión, a descomposición térmica (350 - 800 ºC), dando lugar a una fracción líquida (bioaceite), carbón y gas. El bioaceite se puede fraccionar y procesar con distintas tecnologías con el objetivo de obtener una amplia gama de productos finales de alto y bajo valor añadido, pudiendo utilizarse para generación directa de calor y energía, producción de biocombustibles o para generación de productos químicos de base biológico. Para esta última aplicación ha de tenerse en cuenta que, el aceite se puede fraccionar en lignina pirolítica (para resinas), azúcares pirolíticos (fuente de carbono en procesos de fermentación) y una fase acuosa rica en compuestos orgánicos de menor peso molecular, como el ácido acético [23,25].

Tras la hidrólisis de sacarosa, almidón y celulosa se pueden realizar procesos pertinentes a la plataforma de azúcares de seis carbonos (C6). También se producen azúcares de cinco carbonos (C5) a partir de las hemicelulosas. Los monosacáridos se pueden procesar por la vía química o biológica para la obtención de combustibles, productos químicos, alimentos o piensos [23,26].

En la plataforma de aceites, los triglicéridos se pueden transesterificar (ésteres de alquilo y glicerol) o ser descompuestos en glicerol y ácidos grasos. Se puede obtener así biodiesel, productos químicos (agentes tensioactivos de jabones, detergentes, resinas, linóleos, aceites, lubricantes, fluidos hidráulicos…), nafta o plásticos de base biológica [23].

La digestión anaerobia de la biomasa (estiércol, residuos de plantas de procesado de alimentos…) en la plataforma de biogás da lugar a metano (CH_4), en un 45 -75 %, dióxido de carbono (CO_2), sulfuro de hidrógeno (H_2S) y vapor de agua, principalmente. Este biogás puede usarse como fuente directa de calor y energía, como gas natural renovable, combustible para el transporte o como precursor de productos químicos de base biológica [23,26].

En la plataforma de soluciones orgánicas se elimina el agua de la biomasa fresca húmeda (hierba, tréboles, cereales inmaduros…), obteniendo, por un lado, una solución orgánica rica en nutrientes (carbohidratos, proteínas, aminoácidos, ácidos orgánicos, minerales, hormonas y enzimas) y, por otro lado, una pasta lignocelulósica rica en fibras

que se puede utilizar como pellets, materia prima en otras plataformas o procesadas a productos de fibra [23].

La plataforma de lignina convierte este compuesto en múltiples productos químicos de elevado valor añadido como BTX (Benceno, Tolueno, Xileno), fenol, vainillina, fibra de carbono, floculantes o polímeros. Además, se puede generar directamente energía y calor a partir de la combustión. También se puede someter a procesos de gasificación, pirólisis o licuefacción hidrotérmica [23,27].

El hidrógeno de la plataforma del mismo nombre se puede obtener a partir de reacciones de *Water-Gas Shift*, electrólisis del agua o procesos de fermentación. Posteriormente, se empleará para generación de biocombustibles, productos químicos de alto valor añadido o fertilizantes, entre otras aplicaciones [23].

Dependiendo del grado de integración de la biorrefinería, la electricidad y el calor generados pueden ser empleados internamente, en la misma instalación, o ser vendidos a la red de suministro [21,23].

1.1.3 Clasificación de las biorrefinerías

La biorrefinerías con comúnmente clasificadas en tres categorías, en función del tipo de materia prima de partida y la variedad de productos que generan:

-Generación 1: Utilizan biomasa con potencial como fuente de alimentación procedente de cultivos con alto contenido de azúcar (caña de azúcar, remolacha azucarera), rastrojos de maíz, paja de trigo, maíz. Generan bioaceites, biocombustibles y biogases [18,28].

-Generación 2: Emplean materias primas que no son fuente de alimento, pudiendo ser lignocelulósicas, residuos de agricultura, industria, residuos de madera (silvicultura) y hierbas. Generan biocombustibles, biomasa, bioaceites, calor, energía, electricidad y productos bioquímicos [18,28].

-**Generación 3:** Emplean organismos acuáticos (microalgas, macroalgas) como materia prima. Producen biocombustibles, bioaceites, biogases, calor, potencia, electricidad, fertilizantes y productos bioquímicos [18,28].

Otro de los criterios para clasificar este tipo de instalaciones es el producto principal que se busca obtener: biorrefinerías orientadas a la obtención de energía (calor y electricidad y/o biocombustibles) u orientadas a la generación de bioproductos químicos, biomateriales, alimentos y piensos [21].

1.1.4 Estado de desarrollo de las biorrefinerías

A pesar del alto potencial de las biorrefinerías como cimiento para el desarrollo de la bioeconomía y el cumplimiento de los ODS, estas instalaciones todavía no han sido suficientemente implementadas a nivel mundial. Incluso en países desarrollados, pocas biorrefinerías reales están operando a nivel industrial en comparación con los procesos basados en petróleo existentes. Sin embargo, se espera que el mercado de las biorrefinerías crezca desde USD 141.800 millones en 2022 a USD 210.300 millones en 2027 [29,30].

En 2022 se encontraron en activo 1.312 biorrefinerías a escala internacional. Cabe destacar que 637 de estas instalaciones se ubicaron en Estados Unidos, 100 se situaron en Francia, 78 en Alemania, 58 en China, 56 en Países Bajos y 42 en Suiza [31].

Tal y como se muestra en la Figura 1.3, principalmente se empleó biomasa cultivada con el propósito de su uso en estas infraestructuras. Sin embargo, el porcentaje de biomasa procedente de residuos cada vez es más elevado y, en 2022, ya constituyó aproximadamente el 35 % de la total utilizada. La biomasa más cultivada fue aquella rica en almidón, seguida de la oleaginosa y la rica en azúcares. La biomasa lignocelulósica de las tierras de cultivo y la biomasa lignocelulósica de la silvicultura también fueron importantes materias primas de biomasa primaria. La principal materia prima de biomasa secundaria fueron residuos orgánicos no procedentes de la agricultura, silvicultura ni reciclado de productos de base biológica [31].

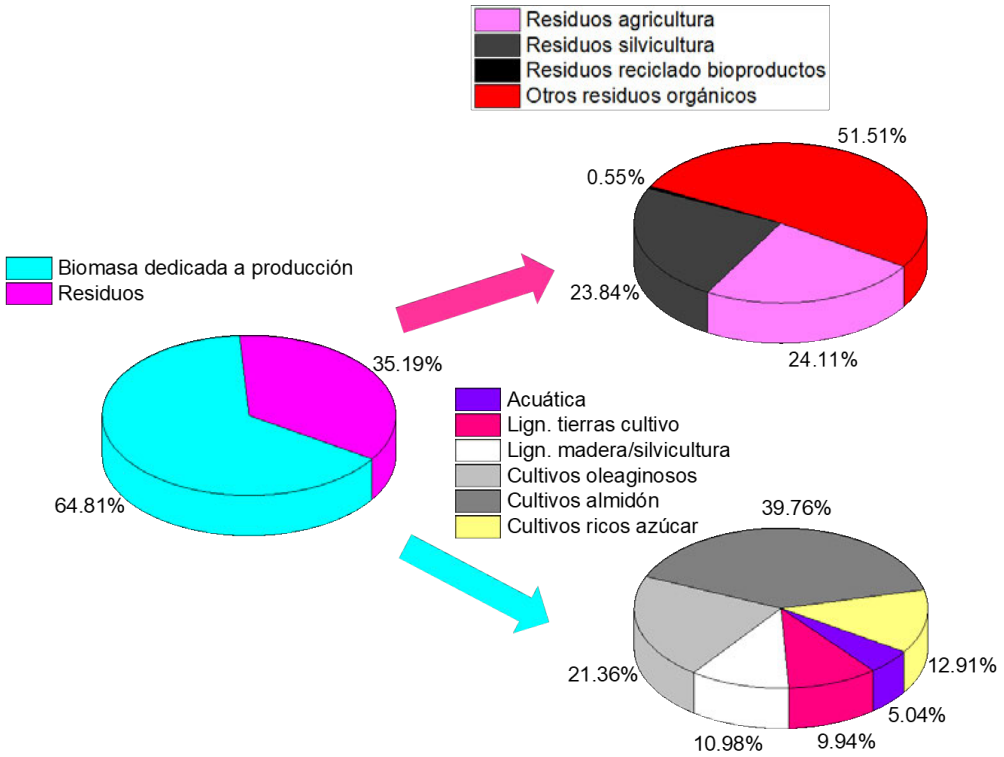

Figura 1.3. Distribución de los tipos de biomasa utilizados como materia prima en las biorrefinerías a nivel global en 2022.

En la Figura 1.4 se puede observar que, en las plantas biorrefineras, se llevaron a cabo, mayoritariamente, conversiones bioquímicas, entre las cuales cabe destacar la fermentación y, en menor medida, los procesos enzimáticos. La conversión catalítica ocupó el segundo lugar debido al extendido empleo de procesos de conversión catalítica, esterificación e hidrogenación, en particular. El tercer tipo de proceso más utilizado son las conversiones mecánicas y termoquímicas, destacando las tecnologías de extracción, separación, gasificación y pirólisis. Por otro lado, se puede apreciar la prevalencia de la generación de energía como objetivo principal en este tipo de instalaciones, especialmente en forma de biocombustible. También se dedican considerables esfuerzos en la producción de químicos (especialmente productos de plataforma y farmacéuticos) y materiales (polímeros y fibras en mayor medida) [31].

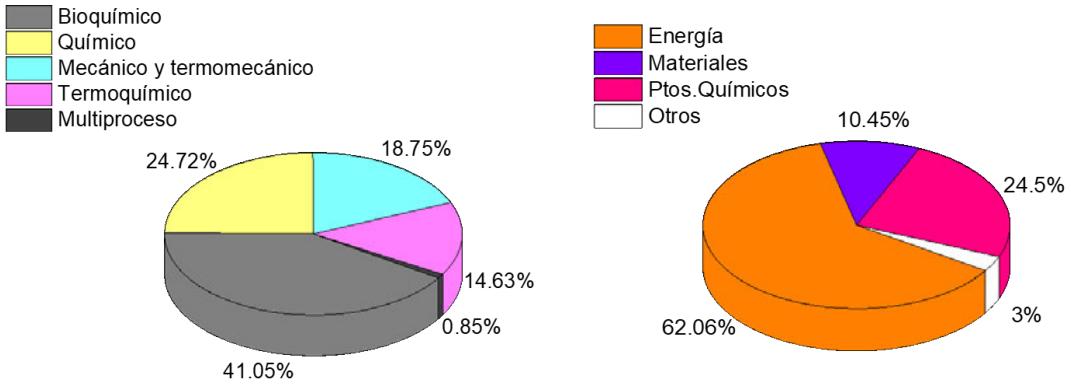

Figura 1.4. Distribución de procesos de conversión y productos generados en las biorrefinerías a nivel global en 2022.

En la Tabla 1.1 se muestra un compendio de información relevante a las biorrefinerías que se encontraban en activo en 2022 en Europa, presentando algunas de las más notables. Entre los países de este continente que cuentan con instalaciones biorrefineras, cabe destacar Francia, Alemania, Países Bajos, Suecia e Italia debido a su elevado número de plantas operativas tanto a nivel comercial como de planta piloto [21,31]. En Austria, aunque la mayor parte de su suministro energético proceda de la importación, en torno a un 51,5 % de la producción nacional procede de biocombustible y residuos. En 2018, el 14 % del consumo energético alemán se produjo a partir de energías renovables y, para 2050, se espera que la biomasa doméstica (de la agricultura, de la madera y de los desechos) contribuya sustancialmente al suministro de energía de este país, pudiendo abastecer el 26 % de las necesidades caloríficas, de electricidad y combustibles. En Italia, la biomasa se utiliza en la producción de biocombustibles y como materia prima para productos químicos intermedios destinados a la producción de plásticos. Entre 2005 y 2018, la producción energética italiana procedente de fuentes renovables se duplicó, cubriendo aproximadamente el 17,8 % del consumo final bruto nacional. En Suecia cada vez cobra mayor importancia la generación de combustibles de base biológica; de hecho, según los datos de 2019, las tres principales fuentes de suministro de energía en Suecia incluían: combustibles nucleares (33 %), biocombustibles (26,5 %) y combustibles fósiles (20,8 %) [31].

Tabla 1.1. Selección de biorrefinerías en activo en Europa.

País	Instalación	Escala	Descripción	Materia prima	Productos
Alemania	Schwedt	Comercial	Biorrefinería de primera generación	Remolacha azucarera, granos	Energía y materiales
Alemania	UPM Leuna	Comercial	Biorrefineria lignocelulósica	Madera	Materiales y productos químicos
Austria	AustroCelBioref inery - Hallein	Comercial	Biorrefinería lignocelulósica	Residuos de celulosa	Bioetanol
Austria	Lenzing AG	Comercial	Biorrefinería lignocelulósica	Ligenocelulosa de agricultura o bosques	Pulpa, bioenergía, ácido acético, furfural, magnesio, lignosulfonato, sulfato de sodio, xilosa
Austria	Lignozellulose - Biorefinery (LCF Biorefinery) - Vienna	Planta piloto	Biorrefinería lignocelulósica	Residuos de cereales, biomasa forestal, residuos de papel y celulosa	Nanolignina, bioactivos, ácidos orgánicos, eritritol, fibras
Dinamarca	Daka Ecomotion	Comercial	Biorrefinería de segunda generación	Residuos de matadero y de agricultura	Biodiesel
Dinamarca	Renescience by Orstred	Demo	Separación enzimática de corrientes residuales sin clarificar en fracciones orgánicas útiles	Residuos municipales sólidos y otras corrientes residuales	Líquidos orgánicos que se pueden convertir en biogás
Dinamarca	Technical University of Denmark	Planta piloto	Gasificación, pirólisis, fermentación, destilación, filtración, secado por aspersión	Biomasa diversa	Diversos bioproductos
España	NEIKER, País Vasco	Semi-industrial	Plataformas de azúcares, lignocelulosa y proteínas	Residuos sólidos urbanos	Bioplásticos y/o termoplásticos
Irlanda	Cellulac Ltd.	Comercial	Empleo de fermentación y bacterias modificadas genéticamente	Materiales lignocelulósicos	Ácido láctico y etilacetato.

Tabla 1.1. Selección de biorrefinerías en activo en Europa (continuación)

País	Instalación	Escala	Descripción	Materia prima	Productos
Italia	Mater Biopymer - Praica, Froisone	Comercial	Construida para generación de PET	Cultivos oleaginosos: palma, coco, colza, girasol, soja	Poliésteres biodegradables
Italia	ENI-Biorefinery di Gela, Sicily	Comercial	Tecnología Ecofining	Biomasa no competitiva con la de alimentación: aceites vegetales, aceite de fritura, grasas de industria cárnica y productos de desecho	Biolubricantes, bioaditivos, ácido azeálico, ácido pelargólico
Italia	Mater-Biotech-Bottrighe, Rovigo	Comercial	Tecnología de fermentación	Azúcares de cultivos ricos en almidón, biomasa lignocelulósica	Químicos, 1,4 biobutanediol
Italia	Reverdia-Cassano Spindola	Planta piloto	Tecnología de fermentación	Trigo y derivados	Ácido succínico
Países Bajos	ACRRES Research Centre Lelystad	Planta piloto	Instalaciones multiusos de biorrefinería	Residuos digeribles y fermentables	Diversos bioproductos
Países Bajos	Emoyro Bioliquids	Comercial	Pirólisis	Biomasa leñosa	Aceite de pirólisis
Países Bajos	Photanol B.V. Corbion Nouryon	Planta piloto	Fotosíntesis, captura de CO_2 por cianobacterias	CO_2	Productos químicos
Suecia	AAK-Dalby	Comercial	Procesado de cultivos producidos en Suecia	Biomasa de cultivos oleaginosos	Grasas vegetales especiales para alimentación y cosmética
Suecia	Pyrocell	Comercial	Pirólisis	Serrín	Bioaceite
Suecia	STQ Gothenburg, Ethanol (Etanolix) Plant(NEOT)	Comercial	Conversión de residuos locales	Residuos biológicos de pastelerías y panaderías	Bioetanol, pienso, biogás

Actualmente, alrededor del 90 % de las materias primas de la industria química de la Unión Europea (UE) para usos no energéticos proceden de recursos fósiles y solo el 10 % de fuentes renovables de carbono. El 6 % de todos los recursos fósiles se dedica solo a la producción de plástico. La creciente preocupación por el cambio climático y la acumulación de plásticos en el medio ambiente propiciará la generación de nuevos materiales de base biológica que sean biodegradables y compostables, en línea con el objetivo general de alcanzar el 30 % de las materias primas de base biológica para la industria química en 2030. Dentro de esta industria de base biológica, se desarrollarán nuevos modelos de biorrefinerías, capaces de transformar diversas biomasas renovables, en lugar de recursos fósiles, en combustibles e intermedios sintéticos para la industria química [31,32].

1.2 LA BIOMASA COMO MATERIA PRIMA PARA BIOPROCESOS

En función de su naturaleza química, la biomasa aprovechable en procesos de fermentación se puede clasificar en las categorías que se presentan en los siguientes apartados [16,33–35].

1.2.1 Biomasa lignocelulósica

La biomasa lignocelulósica la constituyen madera, paja, pastos… Se compone principalmente de celulosa (40-50 %), hemicelulosas (25-35 %) y lignina (15-20 %), aunque también contiene pequeñas cantidades de pectina, proteínas, extractos (azúcares no estructurales, material nitrogenado, clorofila y ceras) y cenizas. Su estructura se muestra en la Figura 1.5. La celulosa es un polímero lineal cristalino de alto peso molecular de β-glucosas unidas entre sí por enlaces glucosídicos β-1,4. Las cadenas poliméricas de la celulosa se agrupan mediante enlaces de hidrógeno y de van der Waals, lo que da lugar a una alta resistencia al ataque biológico. La producción anual de celulosa es de 1,5 billones de toneladas, por los que constituye un recurso ilimitado para la biorrefinería. La hemicelulosa es un polímero amorfo y ramificado (ramificaciones cortas) de cinco azúcares de carbono (xilosa y arabinosa) y azúcares de seis carbonos (galactosa, glucosa y manosa) junto con sustituyentes de ácidos urónicos (por ejemplo, ácidos 4-O-metilglucurónico, D-glucurónico y D-galacturónico). Los monosacáridos están unidos entre sí por enlaces glucosídicos β-1,4 y, a veces, por enlaces glucosídicos

β-1,3. La lignina es el compuesto de alto peso molecular más abundante en la naturaleza. Es un polímero amorfo y tridimensional compuesto por tres unidades diferentes de fenilpropano metoxilado (alcohol coniferílico, alcohol sinapílico y alcohol cumarílico) que están unidas entre sí por diferentes tipos de enlaces C-C y C-O [16,36].

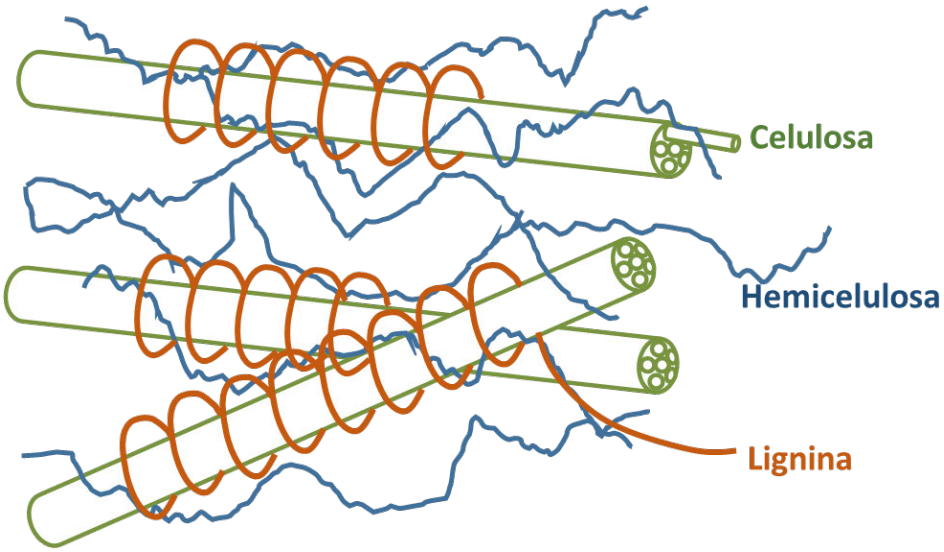

Figura 1.5. Estructura de la biomasa lignocelulósica.

La **celulosa**, polisacárido de elevada resistencia a la hidrólisis, se trata mediante procesos ácidos o enzimáticos, tal y como se explica a continuación:

-Cuando se realiza con ácidos diluidos se pueden producir compuestos no hidrolizables a glucosa (celulosa modificada), glucosa e hidroximetilfurfural (HMF). En el caso de emplear ácidos de elevada concentración a temperaturas moderadas, tiene lugar la ruptura de los puentes de hidrógeno entre las moléculas de los polímeros, acabando con la cristalinidad y minimizando las reacciones de degradación [37–39].

-La hidrólisis enzimática eficiente se consigue mediante la acción sinérgica de endoglucanasas, exoglucanasas y celobiasas, tras haber realizado algún otro pretratamiento que facilite la accesibilidad de las enzimas. Las endoglucanasas (β-(1,4)-glucano-glucanohidrolasa) actúan en los enlaces internos de la celulosa de baja cristalinidad. Las exoglucanasas (β-(1,4)-celobiohidrolasa) liberan celobiosa tras la ruptura de los enlaces extremos de la celulosa amorfa, celulosa cristalina y oligosacáridos de glucosa. La celobiasa (β-(1,4)-glucosidasa) libera glucosa de los extremos no reductores de los oligosacáridos de cadena corta [37,40].

-Los líquidos iónicos, sales cuya temperatura de fusión se encuentra por debajo de 100 ºC, pueden emplearse a temperaturas moderadas para disolver rápidamente la biomasa lignocelulósica o simplemente desestructurarla, incrementando el rendimiento de posteriores tratamientos [37,41].

Para la **solubilización de las hemicelulosas** se pueden llevar a cabo distintos tipos de tratamiento, obteniendo oligómeros o monosacáridos:

-La autohidrólisis con agua a 150-230 ºC es un proceso por el que se produce una despolimerización provocada por la acción catalítica de los iones hidronio y de los compuestos generados en el proceso, dando lugar oligómeros, azúcares, productos de descomposición de monosacáridos y ácido acético. Ha de tenerse en cuenta que, en presencia de proteínas y aminoácidos, los oligosacáridos y monosacáridos pueden experimentar reacciones de Maillard, generando alcoholes, cetonas, aldehídos o furfurales, entre otros compuestos [37–39].

-La explosión con vapor consiste en la inyección de vapor a altas temperatura durante tiempos cortos seguido de una despresurización rápida, propiciando la separación de las fibras e hidrolizando los grupos acetilo de las hemicelulosas. Además, se logra romper los enlaces lignina-hidratos de carbono [37–39].

-En la hidrólisis ácida se rompen las unidades estructurales de los polisacáridos gracias al ataque de los iones hidronio a los enlaces éster. Es habitual la realización de prehidrólisis ácidas que incrementen la susceptibilidad de las celulosas a la hidrólisis [37,39].

-Los tratamientos alcalinos permiten la solubilización de las hemicelulosas, de la lignina y la saponificación de ésteres. A diferencia de los tratamientos anteriores, estos tienen una mayor eficacia a la hora de solubilizar la lignina en relación a la celulosa [37,39].

-También se puede llevar a cabo tratamientos enzimáticos con hemicelulasas. Sobre los xilanos se actúan con las xilanasas y sobre los mananos con las mananasas. Las xilanasas se emplean para la hidrólisis de enlaces xilosídicos, entre ellas se distinguen:

las endoxilanasas (endo-1,4-β-xilanasa y endo-1,3-β-xilanasa) para la ruptura de enlaces glicosídicos en la cadena de xilano y las exoxilanasas (exo-1,4-β-xilosidasa y exo-1,3-β-xilosidasa) que liberan D-xilosas gracias a la hidrólisis de los extremos no reductores de los xilanos. En el caso de que el sustrato tenga ramificaciones será preciso emplear enzimas para la hidrólisis de cadenas laterales, tales como α-D-glucuronidasa, arabinasas, acetil xilano esterasa, ácido ferúlico esterasa y ácido p-cumárico esterasa. Para la liberación de D-manosa se necesitan: endomananasas (endo-1,4-β-mananasa) para la ruptura de los enlaces internos de las cadenas principales, exomananasas (β-manosidasa) que actúen sobre las unidades de manosa unidas al extremo no reductor de los oligosacáridos y β-glucosidasas (1,4-β-D-glucosido glucohidrolasa) para la 1,4-β-glucopiranosa. Para las ramificaciones de las cadenas de mananos se emplean α-galactosidasa y acetilmanano esterasa [37,39,40].

En los procesos de **despolimerización y solubilización de la lignina**, se logra separar la celulosa en la fase sólida, pero la hemicelulosa puede resultar degradada. Cabe destacar los siguientes:

-Empleando sosa se consigue una deslignificación poco profunda, pero se evita la introducción de azufre en el proceso [37,42].

-En el caso de emplear sulfito y sales amónicas o cálcicas, se logra solubilizar con gran eficacia las hemicelulosas y la lignina [37,42].

-En el método Kraft, o de deslignificación con sulfato, se emplea Na_2S y NaOH bajo condiciones agresivas, dando lugar a la "lejía negra" y degradando considerablemente la lignina y las hemicelulosas [37,42].

-El método organosolv se realiza bajo condiciones suaves por acción un disolvente (etanol, acetona…) para conseguir una deslignificación selectiva y una lignina que ha sufrido pocas modificaciones químicas [37,43].

1.2.2 Biomasa rica en triglicéridos

Entre la biomasa rica en triglicéridos se encuentran aceites vegetales, grasas animales, aceites de cocina usados y aceites de microalgas. En los triglicéridos una molécula de glicerol está unida a tres moléculas de ácidos grasos (cuya composición varía según la fuente y el origen geográfico) mediante enlaces éster. Los aceites vegetales están

compuestos de ácidos grasos C8-C24, siendo la mayoría ácidos grasos C16 y C18. La composición de ácidos grasos de los aceites de microalgas es algo más amplia en comparación con los aceites vegetales, pudiendo ser tanto más ligeros o de mayor peso molecular. Las grasas animales suelen estar constituidas por ácidos grasos saturados de alto peso molecular [16,35].

En las biorrefinerías, la biomasa rica en triglicéridos se suele someter a procesos de pirólisis para la obtención de bioaceite, transesterificación para la obtención de biodiesel o incluso fermentación para la generación de bioetanol, biometanol, hidrógeno, ácidos carboxílicos… Para la realización de procesos de pirólisis o transesterificación no se suelen precisar complejas etapas de pretratamiento. Sin embargo, tras la extracción de aceites, la biomasa restante, que puede ser empleada para procesos de fermentación, sí debe someterse a los pretratamientos propios de la biomasa lignocelulósica o de la biomasa rica en sacarosa y almidón [44–47].

1.2.3 Biomasa rica en sacarosa y almidón

Dentro de esta categoría, se puede distinguir tanto aquella biomasa que contiene sacarosa, como en el caso de la remolacha azucarera, sorgo dulce y la caña de azúcar, como aquella rica en almidón, como el trigo, el maíz o la cebada. La sacarosa es un disacárido compuesto por dos monosacáridos C6 diferentes: α-glucosa y β-fructosa, unidos entre sí por un enlace glucosídico α-1-fructosídico β-2. El almidón es un polímero de α-glucosa unido por enlace α-1,4 glucosídico (como en la amilosa) y enlace α-1,6 glucosídico (como en la amilopectina). El almidón generalmente se compone de 20 a 25 % en peso de amilosa y de 75 a 80 % en peso de amilopectina [16,48,49].

Para poder llevar a cabo la fermentación de la **biomasa rica en almidón**, la materia prima se somete a cribado, molienda y, posteriormente, a hidrólisis para la ruptura de las cadenas de almidón y obtener jarabe de glucosa. Para ello, el proceso de molienda húmeda fue el más utilizado hasta la década de 1990 pero, hoy en día, la molienda seca es dominante debido a sus menores costes. La biomasa se muele en seco hasta convertirla en polvo y se mezcla con agua para formar un puré, en el que se agregan enzimas licuadoras (amilasa) para descomponer el almidón en azúcares simples. Luego, el puré se calienta para evitar la contaminación bacteriana y se procede a la etapa de sacarificación,

en la que el almidón licuado se hidroliza a glucosa con enzimas sacarificantes (glucoamilasa). En la molienda húmeda, la biomasa se sumerge en agua y ácido sulfúrico diluido antes de molerla en un puré para facilitar la segregación de sus componentes. Posteriormente se separa el gluten, la fibra y el almidón mediante separadores centrífugos, de malla y ciclones. El almidón se envía al proceso de sacarificación [50,51].

A diferencia de la biomasa rica en almidón, las **materias primas ricas en sacarosa** no requieren el paso de sacarificación, lo que simplifica el proceso de producción. Por lo general, tras una limpieza para eliminar impurezas, la biomasa se prepara para la extracción de azúcares. Este proceso se realiza en molinos, en los que normalmente se adopta el proceso de remojo de la biomasa con agua entre moliendas para recuperar la máxima cantidad de sacarosa de la materia prima o en tambores rotatorios en los que se aplica agua caliente para extraer los azúcares. Al jugo extraído se le añade agua de cal para corregir el pH, permitiendo la coagulación de la materia coloidal y la precipitación de las impurezas. Posteriormente se calienta para evitar contaminaciones y se decanta en tanques para separar las impurezas con la mínima pérdida de nutrientes. Luego, el líquido purificado se concentra para aumentar su contenido de azúcares totales [50,51].

1.2.4 Biomasa rica en proteínas

Algunas de las fuentes tradicionales de proteínas son la soja, el trigo, el maíz, la leche, el colágeno, la queratina, los guisantes o la sangre. Sin embargo, en los últimos años se han diversificado las posibles materias primas, recurriendo a microalgas, proteínas de plantas y hojas (alfalfa, hojas de espinaca, remolacha, jatrofa…), residuos de la industria cervecera (levadura de cerveza gastada) o incluso de la propia industria de los biocombustibles (como las tortas prensadas de colza y girasol) [34,52]. Las proteínas son cadenas de aminoácidos que se pliegan adquiriendo una estructura tridimensional. Los aminoácidos están unidos mediante enlaces peptídicos, es decir, dos moléculas se unen mediante un enlace de tipo covalente CO-NH, perdiendo una molécula de agua y generando un dipéptido. Se producen plegamientos debido a la formación de puentes de hidrógeno entre los átomos que forman el enlace peptídico. La disposición tridimensional la adquieren gracias a la generación de enlaces covalentes entre las cadenas laterales de

los aminoácidos. Cuando se da la conjunción de varias cadenas peptídicas mediante interacciones no covalentes se generan estructuras cuaternarias [53].

En muchos casos, para poder llevar a cabo la liberación de los aminoácidos, la biomasa rica en proteínas puede necesitar someterse a algunos pretratamientos propios de los residuos lignocelulósicos que permitan el acceso de las enzimas a las proteínas a pesar de la presencia de lignina y celulosa en la materia prima. Una vez que la biomasa se ha reducido la resistencia celular, se pueden realizar procesos de hidrólisis proteica, rompiendo los enlaces peptídicos de la cadena de proteínas, dando lugar a polipéptidos y/o aminoácidos libres. Se trata de un proceso que no transcurre en una única reacción, sino que consiste en un conjunto de reacciones en equilibrio simultáneas. La primera reacción es la formación de un complejo enzima-sustrato, posteriormente tiene lugar la ruptura del enlace amídico liberando un péptido y, por último, gracias a un ataque nucleofílico de una molécula de agua, el péptido restante se disocia de la enzima. Estas reacciones se llevan a cabo en condiciones moderadas de temperatura y pH. Para la generación de péptidos de bajo peso molecular mediante ruptura de enlaces peptídicos del interior de la cadena proteica se recurren a endopeptidasas, mientras que, para la generación de polipéptidos y aminoácidos libres, se emplean exopeptidasas que rompan los enlaces en los extremos de las cadenas [54–56].

1.3 EL ÁCIDO SUCCÍNICO

Ácido succínico (SA, ácido 1, 2-etanodicarboxílico, $C_4H_6O_4$), conocido como ácido ámbar o ácido butanodioico, es uno de los compuestos clave en el contexto de las biorrefinerías, siendo considerado uno de los 12 principales productos químicos de plataforma según el Departamento de Energía de los Estados Unidos (*United States Department Of Energy* - US DOE), en otras palabras, se trata de un compuesto con el potencial de ser transformado en nuevas familias de compuestos útiles [57–59].

El ácido succínico puro es un sólido que forma cristales incoloros e inodoros. Tiene un punto de fusión de 185 °C y un punto de ebullición de 235 °C. Es un ácido diprótico. El anión carboxilato se denomina succinato y los ésteres del ácido succínico se denominan succinatos de alquilo. Se trata de un compuesto cuyo peso molecular es de 118,09 g mol^{-1} y densidad de 1,56 g cm^{-3}. Este ácido es combustible y corrosivo, capaz

de causar quemaduras. En forma nutracéutica como aditivo alimentario y suplemento dietético, es seguro y está aprobado por la Administración de Alimentos y Medicamentos (*Food and Drug Administration* – FDA) [60].

1.3.1 Producción química y producción biológica

Tradicionalmente, la producción de ácido succínico se ha realizado a partir de recursos fósiles, principalmente, mediante procesos de hidrogenación catalítica u oxidación parafínica. La oxidación catalítica consiste en la generación de SA oxidando cera de petróleo y otros hidrocarburos parafínicos en presencia de un catalizador de calcio o de magnesio. En la hidrogenación catalítica, el anhídrido maleico, obtenido por oxidación catalítica de hidrocarburos de cuatro carbonos (C4) se somete a una hidrogenación en continuo en condiciones de elevada temperatura y presión, en sistemas catalíticos homogéneos o heterogéneos. A pesar de la madurez de estas tecnologías, cada vez son más criticadas debido al empleo de recursos no renovables y las agresivas condiciones de operación que emplean, con el consecuente consumo de recursos energéticos y emisión de contaminantes [61–63].

Ante la necesidad del desarrollo de una vía alternativa de producción de este ácido sin gran impacto ambiental, se han desarrollado métodos biológicos basados en procesos de fermentación. En la Tabla 1.2 se muestra un resumen de los principales aspectos a tener en cuenta al realizar la comparativa entre este tipo de vías de producción y las químicas tradicionales [64].

Aunque, por la vía biológica, teóricamente un mol de glucosa y dos moles de CO_2 podrían transformarse en dos moles de ácido succínico, en la práctica, los productos secundarios de la fermentación (ácidos acético y fórmico, principalmente) conducen a una disminución del rendimiento. Por este motivo, se están dedicando esfuerzos de investigación en manipulaciones genéticas y mejoras de proceso que permitan reducir la generación de subproductos. Otro de los inconvenientes que presenta este modo de operación son los elevados volúmenes requeridos en los equipos, debido a la alta dilución de sustratos y productos. Además los tiempos de reacción son más prolongados [64,65].

Sin embargo, cabe destacar que la vía de producción biológica cuenta con notables ventajas tales como la fijación de CO_2 en el proceso, la operación bajo condiciones de operación no agresivas, así como la posibilidad de reciclaje y reutilización de desechos como materias primas renovables. Todos estos aspectos cobran especial importancia en este momento en el que existe una preocupación generalizada por el medio ambiente y la búsqueda de industrias "más verdes" [66].

Tabla 1.2. Comparación entre las vías química tradicional y biológica para la producción de ácido succínico.

Tipo de producción	Química	Biológica
Materia prima	Recursos no renovables, petroquímicos	Materias primas de base biológica, carbohidratos
Consideraciones sobre costes	Se espera disminución en la disponibilidad de la materia prima con su consecuente incremento de precios.	Materia prima sin apenas costes, bajas demandas energéticas
Tecnología	Desarrollada y establecida	En desarrollo
Rendimientos/ Productividades	Generalmente elevados	Variable, en ocasiones elevada cantidad de subproductos, medios diluidos, largos tiempos de reacción
Desventajas	Elevada demanda energética, problemas con los desechos de catalizadores	Sensibilidad de los microorganismos, requerimientos nutricionales, difícil recuperación del producto
Imagen pública	Popularidad en disminución	Creciente interés en mejorar las tecnologías actuales y en innovaciones

Las diferencias de costes de producción por ambas vías cada vez son más reducidas debido, principalmente, al aumento en el precio de la materia prima petroquímica. Sin embargo, las materias primas básicas no son los únicos componentes necesarios en las fermentaciones. El costo de la ruta de producción fermentativa y el producto final se incrementa por los nutrientes requeridos, pero principalmente por los costos de recuperación del producto. El precio de los nutrientes, como el extracto de

levadura o la peptona, las fuentes de nitrógeno necesarias para las fermentaciones, es sustancialmente más alto que el precio de los carbohidratos, por lo que debe ser parte de las consideraciones económicas. Se han realizado esfuerzos para reducir el precio del ácido succínico haciendo que los procesos de producción sean más eficientes en términos de rendimiento, productividad, concentración y recuperación del producto, así como mediante un uso eficiente de materias primas más baratas. Por otro lado, el precio del ácido maleico ha aumentado considerablemente en los últimos años. Esto está creando oportunidades para el ácido succínico como sustituto del ácido maleico, especialmente porque la demanda industrial de este último sigue aumentando [64–66].

1.3.2 Aplicaciones del ácido succínico

Desde que Robert Koch demostrara en 1886 la influencia positiva del ácido succínico en el metabolismo, este compuesto se ha empleado en la industria alimentaria como acidulante, aromatizante y edulcorante [57]. Además, el ácido succínico cuenta con multitud de aplicaciones en la industria farmacéutica, siendo también clave para la producción de disolventes verdes, detergentes, inhibidores de la corrosión, surfactantes y quelantes [67,68].

Gracias a su gran reactividad y versatilidad, el ácido succínico se puede emplear como químico de plataforma, dando lugar a compuestos como la succinimida y el anhídrido maleico, que a su vez permiten la obtención de otros compuestos como la hidroxisuccinimida, la succinimida maleica, o incluso otros ácidos carboxílicos (ácidos itacónico, aspártico, maleico o fumárico). La generación de productos químicos intermedios es una de las principales aplicaciones que se espera explotar en la era de la bioeconomía, facilitando el proceso de integración de las biorrefinerías y, por tanto, mejorando su rendimiento y flexibilidad. Otro de sus usos más prometedores es la fabricación de polímeros biodegradables, gracias a la conversión previa de este ácido en dimetil/dietil succinato. Esto a su vez posibilita otra de sus aplicaciones, la generación de solventes (tetrahidrofurano y 2-pirrilodinona) [62,68–70].

En el esquema de la Figura 1.6 se recogen las principales aplicaciones del ácido succínico y sus derivados.

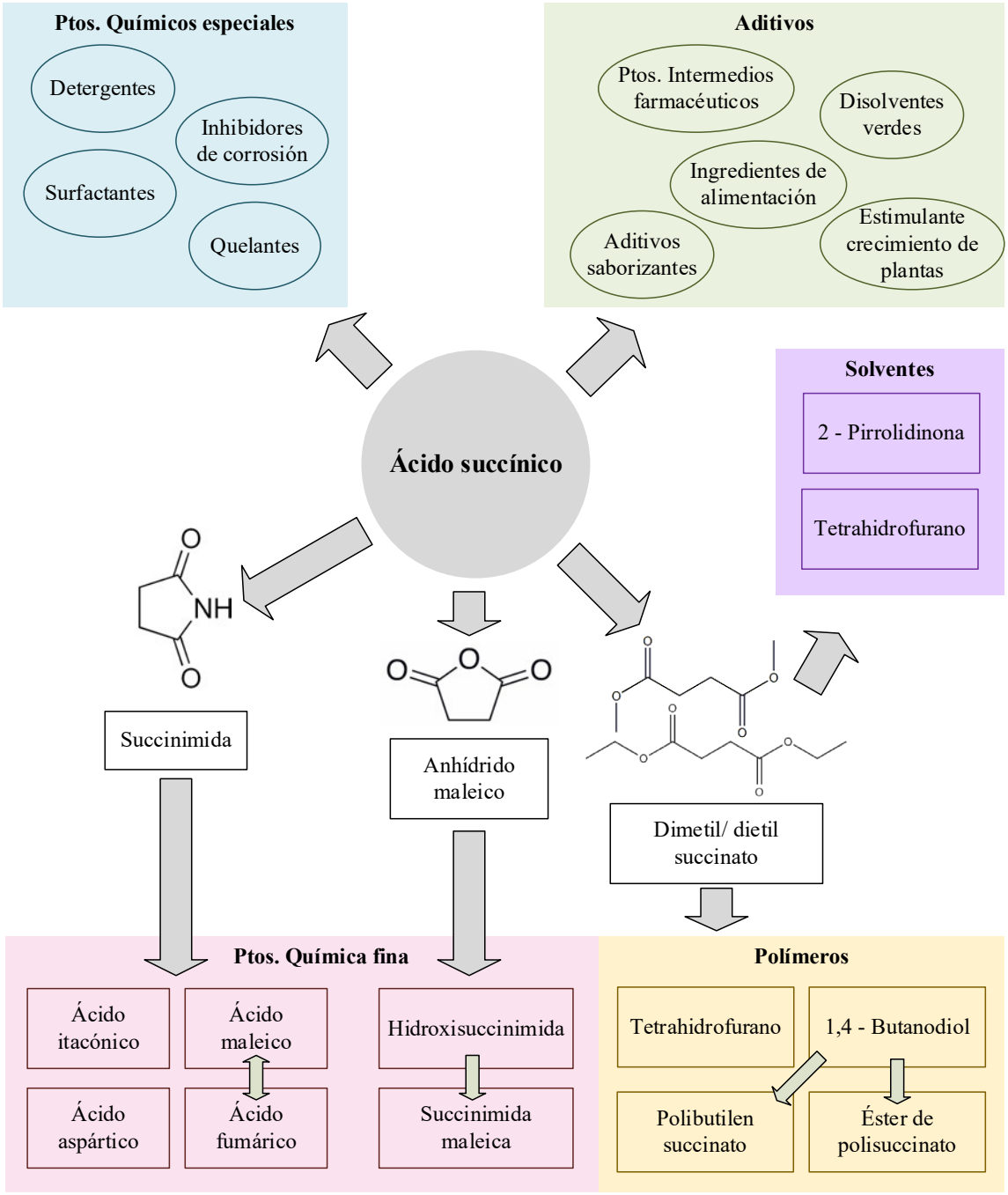

Figura 1.6. Aplicaciones del ácido succínico

1.3.3 Situación actual de mercado del ácido succínico

Debido a la transición hacia la producción de químicos de plataforma y polímeros a partir de ácido succínico de base biológica, así como al uso creciente del ácido succínico en la industria farmacéutica, alimentaria y en las actividades de construcción y desarrollo de infraestructuras, se mantienen perspectivas positivas en relación al crecimiento del

mercado de este compuesto [72]. De hecho, el mercado mundial de ácido succínico se valoró en 160,8 millones de USD (*United States Dollar*) en 2022 y se espera que se expanda a una tasa de crecimiento anual compuesta (CAGR) del 6.5 % entre 2022 y 2032, alcanzando un valor de 301,4 millones de USD [73].

Se prevé que el aumento del uso de ácido succínico a base de petróleo en la producción de poliuretano como alternativa al ácido adípico impulse el crecimiento del segmento de ácido succínico a base de petróleo en los próximos años. En la actualidad, las ventas de ácido succínico a base de petróleo representan un valor de 98,6 millones de USD, que se prevé que aumente a 190,5 millones de USD para finales de 2032. Por otro lado, para entonces se espera que el mercado del ácido biosuccínico aumente a una CAGR del 6 % El aumento de la conciencia sobre la salud y las preocupaciones ambientales por parte de los gobiernos de todo el mundo son factores que impulsan firmemente la demanda de ácido succínico de base biológica. Se espera que el mercado de ácido succínico procedente de la biomasa represente una cuota de mercado del 36,8 % y unos ingresos de 110,8 millones de USD a finales de 2032 [73].

Por otro lado, cabe destacar que, en los últimos años, el coste de ácido succínico de base biológica ha continuado reduciéndose y equiparándose al procedente de recursos fósiles. En 2015 el precio de mercado de este ácido producido por la vía biológica fue de 2,86 $USD \cdot kg^{-1}$, mientras que el obtenido por la vía tradicional fue de 2,50 $USD \cdot kg^{-1}$ [57,74]. Sin embargo, debido al gran interés en los procesos biotecnológicos para SA, actualmente ya se han desarrollado e incluso implementado a nivel industrial algunos procesos económicamente competitivos, produciendo SA con un precio de mercado entre 2,00 y 2,50 $USD \cdot kg^{-1}$ [75].

Europa dominó el mercado en 2021 y representó la mayor parte de los ingresos de más del 35,5 % en 2021. Esto es debido, principalmente, al aumento de la demanda en los sectores de la salud y la agricultura en la región junto a la estricta imposición normativa sobre el uso de determinados productos químicos peligrosos que amenazan el medio ambiente y los seres humanos, lo cual brinda una gran oportunidad para el desarrollo de la industria del ácido succínico de base biológica. En Europa, el ácido succínico está muy extendido en la industria cosmética y de cuidado personal, albergando muchas marcas de cosméticos, como Unilever y P&G. La sólida presencia de fabricantes

de aviones en varias partes de Alemania y el Reino Unido ha conducido al crecimiento del mercado de ácido succínico en el sector aeroespacial. América del Norte representó la segunda fuente de ingresos más grande del mercado global del ácido succínico en 2021. La producción de ácido succínico a partir de la conversión microbiana de materias primas renovables está logrando despertar interés en América del Norte como un medio para lograr el desarrollo sostenible en esta era de escasez de petróleo. En los últimos años se está observando un intenso desarrollo del mercado en la región de Asia-Pacífico, favorecido por la disponibilidad de mano de obra de bajo costo, siendo China el país con la mayor capacidad de producción entre todos los demás de la región [76–78].

El mercado global es muy competitivo debido a la presencia de varias empresas multinacionales que intentan obtener una ventaja competitiva con una sólida cartera de productos, diversificación, red de distribución y estrategias comerciales, como asociaciones, empresas conjuntas y expansiones de capacidad. En los últimos años las empresas dedicadas a la producción de ácido succínico de base biológica han experimentado fuertes periodos de desarrollo y crecimiento, así como valles ocasionados, principalmente, por las fluctuaciones del precio del petróleo [76,79]. Establecida en 2008, BioAmber fue la primera en comercializar ácido biosuccínico después de perfeccionar su tecnología en una pequeña planta en Francia. En 2015, BioAmber y Mitsui, en colaboración, pusieron en funcionamiento una planta de 30 ktpa o kt/año en Sarnia, Canadá, y poco después informaron unos ingresos de 2,5 millones de USD durante un trimestre. Antes de finales de 2013, Myriant, subsidiaria de PTT Global Chemical (PTTGC), inauguró una planta de ácido succínico de 14 ktpa en Luisiana, EE. UU. Reverdia, una empresa conjunta de DSM/Roquette, ingresó al mercado con una planta de 10 ktpa en Italia. Además, con la fundación de una planta de 10 ktpa en España por parte de BASF/Corbian Purac en colaboración con Succinity al año siguiente, el futuro del ácido biosuccínico parecía muy prometedor. Sin embargo, en 2018, el colapso de los precios del petróleo crudo hizo que el costo de las materias primas basadas en petroquímicos fuera significativamente más bajo que las fuentes renovables para productos de base biológica. Con la disminución de las ventas y el regreso de los clientes a sus cadenas de suministro basadas en productos petroquímicos, BioAmber quebró al poco tiempo. Myriant nunca alcanzó la capacidad total de la planta y solo fue capaz producir en lotes. La planta estuvo inactiva en 2016 y fue vendida a Stepan en 2021. El nuevo propietario actualmente está reconvirtiendo las instalaciones para producir

biosurfactantes. Reverdia continuó como una empresa conjunta de DSM/Roquette hasta 2019, cuando se anunció que la empresa se había disuelto y que DSM se convertiría en el licenciante exclusivo para clientes estratégicos. La tecnología había madurado hasta un punto en el que el potencial de implementación era lo suficientemente significativo como para justificar un esfuerzo centrado en la concesión de licencias. La pionera planta de Sarnia de BioAmber fue adquirida por LCY con sede en Taiwán y, para 2021, según se informa, había logrado alcanzar una capacidad de 18 ktpa. El objetivo de LCY es llegar a 30 ktpa para 2023 [76,80].

1.4 PRODUCCIÓN BIOTECNOLÓGICA DE ÁCIDO SUCCÍNICO

1.4.1 Microorganismos productores de ácido succínico

Casi todas las células microbianas, vegetales y animales pueden generar SA. Sin embargo, a lo largo de los años se ha observado que los organismos más adecuados para la producción de este compuesto son los hongos y las bacterias [59]. Algunos hongos como *Aspergillus niger*, *Penicillium viniferum*, *Yarrowia lipolytica, A. fumigatus, Byssochlamys nuvea, Lentinus degener, Paecilomyces varioti,* y la levadura *Saccharomyces cerevisiae* generan AS como subproducto de su metabolismo en condiciones aeróbicas y/o anaeróbicas [62,65]. Aunque se han dedicado considerables esfuerzos en la investigación de la producción de ácido succínico mediante la acción de estos microorganismos, su uso se ha encontrado limitado debido a las excesivas dificultades presentadas tanto en la fermentación como en los procesos de separación y purificación [59,81]. Las cepas bacterianas más investigadas son *Actinobacillus succinogenes*, A*naerobiospirillum succiniproducens*, *Escherichia coli* recombinante, *Corynebacterium glutamicum* y *Mannheimia succiniciproducens*, [65,81–84]. Hasta la fecha, las bacterias aisladas del rumen del ganado (*A. succinogenes* y *M. succiniciproducens*) son las que se considera de mayor potencial, debido a su capacidad de generar naturalmente ácidos dicarboxílicos C4 durante la digestión pregástrica de los polisacáridos [63,65,81,85].

Estos microorganismos del rumen producen SA a través del llamado ciclo del ácido tricarboxílico (*TriCarboxylic Acid* - TCA), que se muestra en la Figura 1.7. Se puede distinguir la ruta de glicólisis, una serie de reacciones orientadas a la generación

energética que transforman la glucosa en dos moléculas en fosfoenolpiruvato (*PhosphoEnolPyruvate* - PEP). Para activar la ruta de producción de ácido oxalacético a partir de PEP, se requiere alimentación de CO_2. El oxalacetato se reduce posteriormente en una serie de etapas hasta dar lugar a SA. Por otro lado, se observa la ruta de la pentosa fosfato, que permite el aprovechamiento de azúcares 5C. En esta vía tiene lugar una primera fase oxidativa, formándose pentosa ribulosa-5-fosfato a partir de glucosa-6-fosfato. Posteriormente, en la fase no oxidativa, se encadenan una serie de reacciones para la formación de gliceraldehído-3-fosfato [75,81]. En función de la ruta de reacción que muestra el TCA, teóricamente para obtener 1 mol de SA serían necesarios 2 moles de nicotina-mida adenina dinucleótido reducido (*Nicotinamide adenine dinucleotide* H - NADH) y 1 mol de CO_2. En cuanto a los azúcares, serían necesarios 0.5 mol de glucosa, 0.6 mol de xilosa o 1 mol de glicerol para generar otro mol de succinato [86].

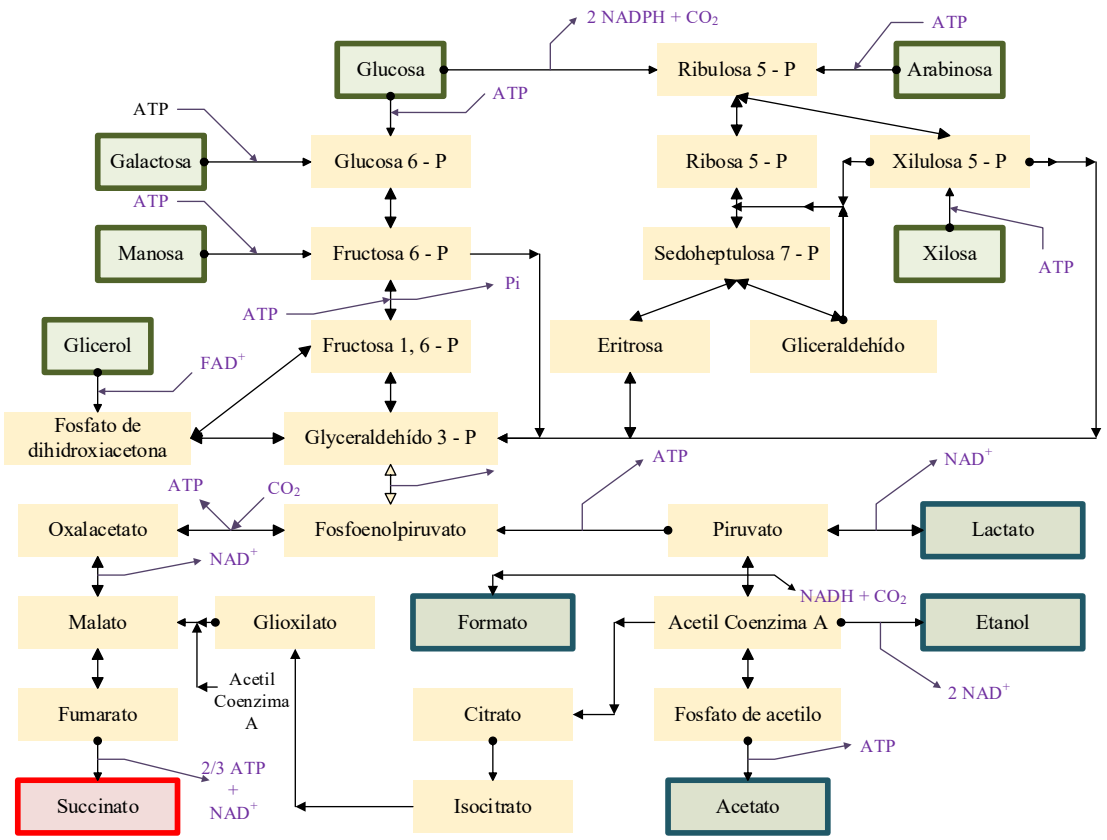

Figura 1.7. *Vías metabólicas en microorganismos productores de ácido succínico como A. succinogenes, A. succinoproducens, M. succiniproducens y E. coli modificada. Trifosfato de adenosina (Adenosine TriPhosphate -* ATP), din*ucleótido de flavina y adenina (Flavin Adenine Dinucleotide FAD*$^+$*), nicotinamida adenina dinucleótido (Nicotinamide Adenine Dinucleotide - NAD*$^+$*), nicotinamida adenina dinucleótido reducido (NADH), nicotinamida adenina dinucleótido fosfato reducido (Nicotinamide Adenine Dinucleotide Phosphate* reduced - *NADPH).*

Cabe resaltar las diferentes características fisiológicas de las bacterias productoras de SA. *A. succiniproducens*, *A. succinogenes* y *M. succiniproducens* producen naturalmente SA como el principal producto de la fermentación en presencia de CO_2 a través de la vía de la carboxinasa PEP [86,87]. Se ha observado que, en el metabolismo de *A. succiniproducens*, la PEP carboxiquinasa (PhosphoEnolPyruvate CarboxyKinase - PEPCK) es la principal enzima fijadora de CO_2 para la generación de oxalacetato. Además, la conversión de piruvato en acetil-CoA depende de la piruvato-ferredoxina oxidorreductasa. Por lo tanto, el control del pH y la concentración de CO_2 son críticos en las fermentaciones con *A. succiniproducens*, ya que la PEPCK y la piruvato-ferredoxina oxidorreductasa son fuertemente dependientes de estos parámetros. Su optimización conduciría a un aumento en la productividad del ácido succínico y una reducción en la generación de subproductos (ácido láctico, ácido acético y etanol) [83,87]. *A. succinogenes* y *M. succiniproducens* comparten muchos aspectos de su metabolismo. En ambos casos, los principales productos de fermentación son los ácidos succínico, acético y fórmico. Sin embargo, *A. succinogenes* también puede generar etanol como subproducto, mientras que *M. succiniproducens* produciría ácido láctico. Durante el metabolismo de ambas cepas se forma ácido oxalacético gracias a la acción de PEPCK y posteriormente se reduce a succinato por la vía C4. Aunque probablemente no sea la enzima principal en este proceso, se ha sugerido *que M. succiniproducens* también puede usar PEP carboxilasa (PhosphoEnolPyruvate Carboxylase - PEPC) para la carboxilación del ácido oxalacético; sin embargo, PEPC no está codificada en el genoma de *A. succinogenes* [86]. Aunque *E. coli* es capaz de producir ácido succínico a través de la rama reductora del ácido tricarboxílico (TCA), no es el producto principal de su fermentación de forma natural, por lo que se ha utilizado la ingeniería metabólica para incrementar la producción [81,86]. Las enzimas PEP, PEPC y carboxiladoras de piruvato se producen en exceso para dirigir el flujo metabólico a la rama reductora de TCA. PEPCK de *A. succinogenes* y piruvato carboxilasa (PYruvate Carboxylase - PYC) de *Lactococcus lactis* o *Rhizobium etli* también se generan en exceso [86]. La cepa de *E. coli* NZN11 es capaz de excretar piruvato mediante el incremento de su carboxilación, sin embargo, no es capaz de un crecimiento fermentativo y cuando se produce en exceso la enzima málica, la producción de succínico se ralentiza en gran medida [83,86]. Gracias a los experimentos sobre la transición de la fase de crecimiento aeróbico a la producción anaeróbica realizados con *E. coli*, se descubrió que, durante el crecimiento aeróbico, se activaba una nueva vía que involucraba la derivación del glioxilato, utilizando menos

poder reductor y complementando la rama reductora de TCA [86]. Con las cepas AFP111 y SBS550MG/pHL413, el objetivo fue eliminar los subproductos de la fermentación y garantizar el flujo de derivación del glioxilato. Sin embargo, ha de tenerse en cuenta que, si se pudiera utilizar un reductor adicional, la ruta del glioxilato sería menos eficiente que en el caso de maximizar el flujo de la rama TCA reductora. *C. glutamicum* en condiciones anóxicas, con carbonato y en ausencia de crecimiento, es capaz de producir ácidos succínico, láctico y acético. En este microorganismo, el ácido oxalacético se produce principalmente gracias a la acción de PEPC con menor influencia de PEPCK y PYC, posteriormente este compuesto da lugar al ácido succínico a través de la rama reductora del ciclo TCA, resultando innecesaria la derivación del glioxilato [71,83,86,87].

1.4.2 Condiciones y tipos de operación empleados

La bibliografía relativa a la producción de ácido succínico de base biológica es muy extensa y de gran variedad. Se han realizado multitud de estudios de optimización de condiciones de operación, así como de formas de operación, empleo de diferentes microorganismos y sustratos que permitieran reducir los costes de la fermentación.

Algunos de los microorganismos más estudiados son *E. coli* modificadas genéticamente y *Y. lipolytica*, con los cuales se ha logrado productividades competitivas con las fermentaciones realizadas con bacterias procedentes del rumen del ganado [88–93]. Sin embargo, es con estas últimas con las que se consigue alcanzar los rendimientos los rendimientos más elevados, destacando *A. succinogenes* entre ellas [94–96].

En los procesos con *A. succinogenes*, la producción de ácido succínico se suele realizar a 33-39 °C, pareciendo haber logrado un consenso de un valor óptimo de 37 ºC. Se busca mantener un pH controlado entre 6.5 y 7. El CO_2 se suele aportar al sistema por insuflación en estado gaseoso o incorporando carbonatos al sistema [70,97–102]. Se han empleado diferentes carbonatos ($NaHCO_3$, K_2CO_3, $CaCO_3$ o $MgCO_3$) a varias concentraciones [103,104], comparándolos con CO_2 gaseoso puro o procedente de biogás a diferentes presiones parciales [105–108] e incluso maximizando su solubilidad al aumentar la presión en el reactor [109,110]. Observando resultados similares empleando $MgCO_3$ en la misma cantidad que la fuente de carbono o exceso de CO_2 gas, especialmente cuando se incrementa la presión del proceso [105–110].

Tal y como se puede apreciar en la Tabla 1.3, los investigadores han estudiado diferentes configuraciones y tipos de reactores, consiguiendo aumentar considerablemente los rendimientos con operaciones tipo discontinuo en serie o *repeated batch* con soporte y semicontinuo o *fed-batch* [111–115], además, gracias a operaciones continuas con formación de *biofilm*, en algunos casos, se ha logrado mejorar drásticamente los valores de productividad [115–118]. Cabe destacar el trabajo de Kim et al. [119], quienes realizaron una fermentación continua de células recicladas para maximizar la actividad biocatalítica, logrando una productividad de 3,86 g L^{-1} h^{-1} de ácido succínico. Sin embargo, los rendimientos y productividades son especialmente altos cuando se trabaja con células inmovilizadas, operando tanto en *repeated batch*, como en el caso de Cao et al. [120], quienes lograron incrementar la producción de ácido succínico prácticamente en un 50 % respecto a la operación en *batch*, como en modo continuo, tal y como lo demostraron Ercole et al. [115], produciendo 36,5 g L^{-1} h^{-1} de ácido succínico con células atrapadas en perlas de alginato

De momento, no parecer haber un consenso respecto a la concentración inicial de sustrato óptima. Luthfi et al. [121] y Salvachúa et al. [70] determinaron una concentración de 60 g L^{-1} de glucosa era la adecuada para maximizar el rendimiento de la producción de succínico. Sin embargo, según las conclusiones de Ferone et al. [99], la concentración de glucosa tenía que ser considerablemente inferior, en torno a 40 g L^{-1}. Estos mismos autores también determinaron un óptimo en la producción a bajas concentraciones de xilosa, alrededor de 5 g L^{-1} mientras, Pateraki et al. [100], utilizando como fuente de carbono una mezcla de azúcares rica en xilosa, obtuvieron mejores resultados a una concentración de azúcares totales de 32,5 g L^{-1}. En la última década, las publicaciones relativas a la producción de ácido succínico a partir de biomasa rica en triglicéridos, almidón, sacarosa o lignocelulósica se han multiplicado, debido al firme interés de reducir costes de producción, abandonando el uso de los azúcares comerciales como fuente de carbono [88,94,121,122].

Tabla 1.3. Compilación bibliográfica de procesos de producción de ácido succínico a partir de distintos microorganismos, fuentes de carbono, nitrógeno y tipos de operación.

Microorganismo	Fuente de C	Fuente de N	Tipo de operación	Condiciones de operación	Rend. ($g\ g^{-1}$)	Prod. ($g\ L^{-1}\ h^{-1}$)	C_{SA} ($g\ L^{-1}$)	Ref.
A. succinogenes NJ113	Glucosa	Extracto de levadura	*Batch*	37 °C, 200 rpm, pH 6,8, CO_2 0,2 L min^{-1}	0,63	0,13	20,8	[123]
A. succinogenes 130Z	Glucosa	Licor maíz fermentado	*Batch*	30ºC, 120 rpm	0,52	0,29	20,6	[124]
A. succinogenes 130Z	Mezcla azúcares hidrólisis lignocelulósica.	Extracto levadura	*Batch*	37ºC, 150 rpm, $MgCO_3$ control pH y fuente CO_2	0,55	0,22	27,0	[99]
A. succinogenes 130Z	Hidrolizado de aceite de palma	Extracto de levadura	*Batch*	37ºC, 200 rpm, pH 6.8, CO_2 0,5 vvm	0,57	1,95	36,5	[121]
A. succinogenes 130Z	Hierba Napier	Extracto de levadura	*Batch*	37 °C, 200 rpm, pH 6,7, CO_2 0,5 vvm	0,58	0,79	17.5	[122]
A. succinogenes 130Z	Mosto de uva	Extracto de levadura	*Batch*	37 °C, 150 rpm, $MgCO_3$ control pH y fuente CO_2	0,67	0,45	43,2	[94]
B. succiniciproducens JF4016	Mosto de uva	Extracto de levadura	*Batch*	37 °C, 150 rpm, MgCO3 control pH y fuente CO_2	0,46	0,35	33,3	[94]
E. coli AFP184	Jarabe de sorgo dulce	Licor maíz fermentado	*Batch*	37ºC, 300 rpm, aireación 1 vvm, pH 6,5	≈ 0,34	1,05	27,0	[88]
Y. lipolytica PGC01003	Glicerol	Extracto de levadura, triptona	*Batch*	28ºC, 600 rpm, pH 6, aireación 2 L min^{-1}	0,25	0,07	7,00	[89]

Tabla 1.3. Compilación bibliográfica de procesos de producción de ácido succínico a partir de distintos microorganismos, fuentes de carbono, nitrógeno y tipos de operación (continuación).

Microorganismo	Fuente de C	Fuente de N	Tipo de operación	Condiciones de operación	Rend. ($g\ g^{-1}$)	Prod. ($g\ L^{-1}\ h^{-1}$)	C_{SA} ($g\ L^{-1}$)	Ref.
Y. lipolytica PGC01003	Glicerol	Extracto de levadura, triptona	Biorreactor de lecho fibroso in situ	28ºC, 600 rpm, pH 6, aireación 3 L min^{-1}	0,45	1,45	53,6	[90]
A. succinogenes UK13	Glucosa	Extracto de levadura, licor maíz fermentado	Reactor continuo de reciclado celular	38°C, 200 rpm, pH 6,8	0,88	3,86	47,7	[119]
Y. lipolytica. PGC62-SYF-Mae	Glucosa	Extracto de levadura, triptona	*Fed-batch*	30ºC, 600 rpm, pH 5,5, aireación 2 vvm	0,37	0,70	101	[114]
A. succinogenes 130 Z inmovilizada en perlas de alginato	Glucosa	Extracto de levadura	Continuo lecho fluidizado	37ºC, pH 6,5, CO_2 10 N L h^{-1}	0,76	35,6	31,0	[115]
A. succinogenes 130 Z inmovilizada en perlas de alginato	Glucosa	Extracto de levadura	*Repeated batch*	37ºC, 150 rpm, $MgCO_3$ fuente CO_2 y control pH	0,49	2,50	43,0	[115]
A. succinogenes 130Z immovilizada en palos de madera	Xilosa	Extracto de levadura, licor maíz fermentado	Continuo	37ºC, pH 6,8	0,68	3,40	29,4	[117]

Sin embargo, hay otras variables de operación que no han sido estudiadas o que apenas se han revisado y cuyos valores fluctúan excesivamente entre las distintas publicaciones, tal y como ocurre con la concentración inicial de biomasa o la velocidad de agitación. Además, hasta el momento, los estudios centrados en la fuente de nitrógeno son escasos y no se ha explorado completamente el impacto que esta variable puede tener en la producción [105,125,126]. Cabe destacar el trabajo de Tan et al. [127], quienes compararon el uso de 15 g L^{-1} de extracto de levadura y licor de maíz fermentado, logrando una cantidad de ácido succínico del 3,7 %, menor que usando extracto de levadura como fuente de nitrógeno y reduciendo en una quinta parte los costos asociados con la fuente de nitrógeno. Xi et al. [128] estudiaron el efecto del uso de licor de maíz fermentado a diferentes concentraciones iniciales obteniendo rendimientos similares en fermentaciones con ambas fuentes de nitrógeno, siempre que la cantidad de licor de maíz duplicara la de extracto de levadura.

1.4.3 Producción a partir de residuos alimentarios

Con el objetivo de desarrollar una bioeconomía basada en biorefinerías capaces de operar de manera competitiva con aquellas que emplean recursos fósiles, resulta imprescindible el empleo de biomasa que, aparte de no servir como fuente de alimento, no implique el incremento de tierras de cultivo para su obtención.

Según la Organización para la Agricultura y la Alimentación (*Food and Agriculture Organization* - FAO) anualmente se desperdician alrededor de 1.300 millones de toneladas de alimentos, para cuyo cultivo se utiliza alrededor del 28 % de la superficie agrícola disponible [129,130]. Además, se prevé que la cantidad de residuos alimentarios por año aumente en un tercio para 2030, en otras palabras, 66 toneladas de alimentos desperdiciados por segundo [131]. Por ello, en la investigación de la producción de ácido succínico, también está cobrando gran interés el empleo de residuos alimentarios como fuente de carbono e incluso de nitrógeno, tal y como se muestra en la Tabla 1.4.

Hasta el momento, la mayor parte de estos trabajos se han realizado mediante operaciones tipo *batch* con *A. succinogenes* como biocatalizador. Se han llevado cabo varios estudios centrados en la sustitución de la fuente de carbono y, en algunos casos la eficiencia del proceso con residuos no se ha visto menguada en relación a su equivalente con azúcares comerciales, como en el caso del trabajo de Oreoluwa Jokodola et al [112],

quienes compararon el desempeño de la fermentación con huesos de aceituna y con xilosa comercial pura, observando que en ambos casos se lograba alcanzar el mismo rendimiento del 27 %.

En cuanto a los pretratamientos, en líneas generales, se ha observado que los procesos enzimáticos conducen a mayores rendimientos durante la fermentación, tanto en los pretratamientos de la fuente de carbono como de la fuente de nitrógeno.

Cabe destacar que apenas se ha profundizado en la sustitución de la fuente de nitrógeno, a pesar de ser uno de los mayores limitantes en estos tipos de proceso debido a sus elevados costes. Cabe subrayar los estudios de Jiang et al. [130], quienes lograron producir succínico a partir de glucosa y levadura de cerveza gasta suplementada con biotina y de Filippi et al. [111], que lograron aprovechar de manera integral los residuos de una bodega, empleándolos tanto como fuente de carbono (tallos y orujo de uva), como fuente de nitrógeno (lías de vino).

Tabla 1.4. *Compilación bibliográfica de procesos de producción de ácido succínico a partir de residuos alimentarios.*

Microorganismo	Fuente de C	Fuente de N	Tipo de operación	Condiciones de operación	Rend. ($g\ g^{-1}$)	Prod. ($g\ L^{-1}\ h^{-1}$)	C_{SA} ($g\ L^{-1}$)	Ref.
A. succinogenes 130Z	Huesos de aceituna	Extracto de levadura	*Fed-batch*	37 ºC, 200 rpm, pH 7, $NaHCO_3$ fuente de CO_2	0,27	0,50	33,7	[112]
A. succinogenes 130Z	Tronco de palma aceitera	Extracto de levadura	*Batch*	37 ºC, 200 rpm, pH 6,8, $MgCO_3$ fuente CO_2	0,47	0,18	10,6	[132]
A. succinogenes NJ113	Glucosa	Levadura de cerveza gastada biotina	*Batch*	37 ºC, 200 rpm, pH 6,8, CO_2 0,5 L min^{-1}	0,68	0,63	47,6	[133]
S. cerevisiae Y2034 y *A. succinogenes* ATCC 55,618	Bagazo de caña de azúcar	Extracto de levadura	*Batch*	37 ºC etapa 1, 33ºC etapa 2, pH 6,8, CO_2 0,3 vvm	0,13	0,36	22,8	[113]
A. succinogenes 130Z	Bagazo de ágave Tequilana	Extracto de levadura	*Batch*	37 °C, 150 rpm, pH 6,7, CO_2 0,3 vvm	0,45	0,57	5,20	[134]
A. succinogenes 130Z	Bagazo de ágave Tequilana	Extracto de levadura	*Repeated batch*	37 °C, 150 rpm, pH 6,7, CO_2 0,3 vvm	0,39	1,32	33,6	[134]
A. succinogenes 130Z	Residuos de cáscara de cítricos	Licor maíz fermentado, vitaminas	*Batch*	37 ºC, 100 rpm, pH 6,8 , CO_2 0,5vvm	0,62	0,69	18,5	[135]
A. succinogenes 130Z	Residuos de cáscara de cítricos	Licor maíz fermentado, vitaminas	*Fed-batch*	37 ºC, 100 rpm, pH 6,8 , CO_2 0,5vvm	0,73	0,45	22,4	[135]
A. succinogenes 130Z	Tallos y orujo de uva	Extracto de levadura	*Fed-batch*	37 °C,180 rpm, CO_2 0,1 vvm	0,67	0,79	40,2	[136]
A. succinogenes 130Z	Tallos y orujo de uva	Lías de vino	*Fed-batch*	37ºC, 180rpm, CO_2 0,1 vvm,	0,64	0,79	37,2	[111]

2. OBJETIVO Y ALCANCE DEL TRABAJO

2 OBJETIVO Y ALCANCE DEL TRABAJO

El objetivo general de esta Tesis Doctoral es el estudio y optimización del proceso de producción de ácido succínico mediante la acción biocatalizadora de *Actinobacillus succinogenes* 130Z, valorizando residuos alimentarios y estudiando la cinética de la fermentación y la transferencia de CO_2 en el caldo de cultivo.

El objetivo general se puede dividir en una serie de subobjetivos que se presentan a continuación:

- Estudio de la preparación del inóculo, incluyendo la posible adaptación de las células a la fuente de carbono que se vaya a utilizar en el proceso, especialmente en el caso del uso de xilosa.
- Determinación de los valores de las condiciones y variables de operación más adecuadas para la realización del proceso como la concentración inicial de biomasa, de fuentes de carbono y nitrógeno, así como de variables con influencia en la transferencia de dióxido de carbono en e medio (velocidad de agitación y caudal).
- Estudio de la posibilidad del empleo de *resting cells* para llevar a cabo este bioproceso, así como la determinación del estado y edad celular óptimos y la posible simplificación del medio de cultivo.
- Determinación del tipo de operación más adecuado (*batch, fed-batch o repeated-batch*) tanto con células en estado de crecimiento como en estado de *resting cells*.
- Empleo de diversos residuos para sustituir tanto la fuente de carbono (residuos de patata y bagazo de cerveza) como la fuente de nitrógeno (levadura de cerveza gastada) en la producción con células en crecimiento. En este subobjetivo se requiere una hidrólisis previa de los residuos a emplear.
- Estudio de formas de operación con hidrolizados de residuos con células en crecimiento (*batch y repeated-batch*).
- Modelización cinética del proceso. Se determinará la influencia del fenómeno de transferencia de CO_2 en la velocidad global del proceso y se llevará a cabo la modelización del bioproceso tratando de incluir la influencia de las variables y fenómenos que en ella influyan. Se realizará tanto para células en estado de crecimiento como en *resting cells*.

3. MATERIALES Y MÉTODOS

3 MATERIALES Y MÉTODOS

En este Capítulo se describen diversos protocolos y procedimientos desarrollados en el laboratorio para la obtención de los resultados experimentales que se recogen en esta Memoria de Tesis Doctoral. Además, se especifican los materiales y equipos empleados, así como las técnicas analíticas y los principales recursos matemáticos aplicados.

3.1 MATERIALES

A continuación, se detalla la composición de los medios de cultivo, la naturaleza de los reactivos utilizados y las características del microorganismo biocatalizador. También se aporta información sobre los residuos empleados como fuente de carbono o de nitrógeno en el proceso de producción y los compuestos y enzimas requeridos para su pretratamiento.

3.1.1 Materias primas

Las materias primas seleccionadas como fuente de carbono rica en glucosa fueron residuos industriales de una planta de producción de patatas fritas. Estos residuos, proporcionados por el grupo empresarial Apex S.A. consisten en trozos de patata cruda que se desechan por sus imperfecciones. Tras su recepción fueron conservados a -18 °C.

Como fuente de carbono rica en xilosa se utilizaron residuos industriales de una planta de producción de cerveza, La Cibeles S.L. Se utilizó bagazo de cerveza que, tras su recepción, se conservó a -18 ºC hasta la realización de su pretratamiento.

Los residuos empleados como fuente de nitrógeno también fueron proporcionados por La Cibeles S.L. En concreto, se recurrió a levadura de cerveza gastada extraída de los grifos inferiores de los fermentadores. También fue conservada a -18 ºC.

3.1.2 Microorganismo

El microorganismo seleccionado para la producción de ácido succínico ha sido *Actinobacillus succinogenes* DSM 22257, perteneciente a la familia Pasteurellaceae, suministrado por la Colección Alemana de Microorganismos y Cultivos Celulares (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*- DSMZ). Según los análisis fenotípicos, se trata de una bacteria facultativa anaeróbica, no móvil, gran negativa y pleomórfica. Se considera no patógeno, siendo de nivel de bioseguridad 1. En función de las condiciones de estrés puede generar biofilm [62,63,137,138].

Al tratarse de un microorganismo originalmente aislado del rumen del ganado, es capaz de generar naturalmente ácidos dicarboxílicos C4 durante la digestión pregástrica de los polisacáridos, en otras palabras, puede llegar a producir elevadas cantidades de ácido succínico a partir de una amplia variedad de fuentes de carbono. Tal y como se mencionó en el apartado 1.4.1, la producción de ácido succínico tiene lugar a través del ciclo del TCA, de modo que, una vez que los azúcares se han transformado en gliceraldehído, el microorganismo convierte el PEP (último producto de la glicólisis) en ácido oxalacético gracias a la acción de la PEPCK, requiriéndose CO_2 para la activación de esta ruta metabólica. Posteriormente, el oxalacetato es reducido en una serie de etapas hasta ácido succínico. Los principales subproductos son los ácidos acético y fórmico [83,139].

3.1.3 Reactivos

En la Tabla 3.1 se enumeran los reactivos comerciales empleados en la realización de la parte experimental de este trabajo, aportándose información acerca de la casa comercial, su pureza, su número CAS (*Chemical Abstracts Service*) y los usos a lo que se destinó.

Tabla 3.1. Listado de reactivos comerciales empleados en la experimentación.

Reactivo	Casa comercial	Pureza (%)	Nº CAS	Usos
Ácido acético	Labkem	99.8	64-19-7	Patrón HPLC
Ácido clorhídrico	Labkem	35-38	7647-01-0	Hidrólisis ácida, ajuste de pH
Ácido fórmico	Labkem	98	64-18-6	Patrón HPLC
Ácido láctico	Panreac	85	70-33-4	Patrón HPLC
Ácido ortofosfórico	Merck	85	7664-38-2	Ajuste de pH
Ácido succínico	Panreac	99	100-15-6	Patrón HPLC
Ácido sulfúrico	Merck	95-98	7664-93-9	Fase móvil HPLC
Agar	Fluka		9002-18-0	Placas petri
Brain Heart Infusion	Merck			Reactivación células liofilizadas
Cloruro de calcio	Labkem	≥ 94	10043-52-4	Formulación medio de cultivo
Cloruro de magnesio hexahidrato	Labkem	≥ 948	7791-18-6	Formulación medio de cultivo
Cloruro de sodio	Labkem	≥ 99.9	7647-14-5	Stock
Etanol	Labkem	≥ 99.8	64-17-5	Patrón HPLC
Extracto de levadura	Labkem	≥ 99.9	8013-01-2	Formulación medio de cultivo
Fosfato dipotásico	Labkem	≥ 99	7758-11-4	Formulación medio de cultivo
Fosfato sódico monohidratado	Labkem	≥ 99	10049-21-5	Formulación medio de cultivo
Furfural	Merck	99	98-01-1	Patrón HPLC
Glicerol	Labkem	≥ 99	56-81-5	Stock
Glucosa	Labkem	99	50-99-7	Sustrato, patrón HPLC

Tabla 3.1. Listado de reactivos comerciales empleados en la experimentación (cont.).

Reactivo	Casa comercial	Pureza (%)	Nº CAS	Usos
Hidrógeno carbonato de sodio	Labkem	≥ 99	144-55-8	Formulación medio de cultivo
Hidróxido de sodio	Labkem	≥ 98	1310-73-2	Ajuste de pH
Hidroximetilfurfural	Merck	≥99	67-47-0	Patrón HPLC
Isopropanol	Labkem	≥ 99.8	67-63-0	Esterilización
Tryptic Soy Broth	Merck			Medio de cultivo
Xilosa	Merck	99	58-86-6	Sustrato y patrón HPLC

3.1.4 Composición de medios utilizados

En las Tablas 3.2 a 3.7 se muestra la composición de los medios utilizados para la conservación del microorganismo a -80 ºC, reactivación y crecimiento celular, así como en los ensayos de producción de ácido succínico tanto con células en estado de crecimiento como de *resting*.

Tabla 3.2. Composición del medio de conservación del microorganismo.

Compuesto	Concentración (g L^{-1})
Cloruro de sodio	9
Glicerol	500

Tabla 3.3. Composición del medio comercial *Brain Heart Infusion*.

Compuesto	Concentración (g L^{-1})
Cloruro de sodio	5
Corazón de res	5
Disodio hidrógeno fosfato	2,5
Glucosa	2
Peptona	10
Sesos de ternera	12,5

Tabla 3.4. Composición del medio comercial *Tryptic Soy Broth*.

Compuesto	**Concentración (g L^{-1})**
Cloruro de sodio	5
Fosfato dipotásico	2,5
Glucosa	2,5
Peptona de caseína	17
Peptona de soja	3

Tabla 3.5. Composición del medio de reactivación celular.

Compuesto	**Concentración (g L^{-1})**
Extracto de levadura	5
Glucosa	10
Hidrógeno carbonato de sodio	10
Fosfato dipotásico	15,5
Fosfato sódico monohidratado	8,5

Tabla 3.6. Composición del Medio de Producción (MPEL) de extracto de levadura y células en crecimiento.

Compuesto	**Concentración (g L^{-1})**
Azúcar (glucosa, xilosa)	X
Cloruro de calcio	0,2
Cloruro de magnesio hexahidrato	0,43
Cloruro de sodio	1
Extracto de levadura	10
Fosfato dipotásico	3
Hidrógeno carbonato de sodio	X

Tabla 3.7. Composición del Medio de Producción con células en *Resting* (MPR).

Compuesto	Concentración (g L^{-1})
Xilosa	20
Fosfato dipotásico	8,7

3.2 EQUIPOS

3.2.1 Equipos para asegurar condiciones de esterilidad

-**Autoclave**. De la casa comercial JP Selecta, modelo Presoclave II 80. Se empleó para esterilizar medios de cultivo y el material necesario gracias al efecto del vapor de agua a una presión de 1,2 bares y una temperatura de 121 ºC durante 15 minutos. Este equipo puede operar en un rango de temperaturas comprendido entre 80 y 134 ºC y 0.62 a 2 bares de presión. Tiene una capacidad de 80 L.

-**Cabina de flujo laminar**. Marca TELSTAR, modelo Mini-V/PCR. Cámara de flujo vertical cuya velocidad de impulsión de aire máxima es de 0,45 m s^{-1}. Consta además de un filtro de aspiración, un filtro absoluto HEPA y una lámpara de luz ultravioleta. Este equipo permite la manipulación del microorganismo sin riesgo de contaminación, pues el aire inyectado a través del impulsor evita la presencia de partículas en suspensión y actúa como barrera contra microorganismos del exterior de la cabina.

3.2.2 Equipos para la conservación y cultivo celular

-**Ultracongelador**. Para la conservación de los microorganismos, se empleó un ultracongelador Sanyo modelo VIP serie MDF U33V, cuyo rango de temperaturas comprende los -50 y -86 ºC.

-Estufa. Marca Selecta, modelo S-202, empleada para la incubación de los microorganismos en placa Petri.

-**Incubadora orbital**. Equipo con calefacción por circulación de aire y movimiento orbital de 25mm de diámetro, utilizado para las incubaciones llevadas a cabo

en botella. Marca Sartorius, modelo Certomat® 1S. Es capaz de operar hasta una temperatura máxima de 60 ºC y un rango de agitaciones de 40-400 rpm.

-**Biorreactor**. La producción de ácido succínico a escala de reactor se realizó mediante un equipo de Sartorius BBI Systems, modelo BIOSTAT® B plus. Se compone de una o dos cubas de borosilicato con camisa, tipo tanque agitado de 1 L o 2 L (Figura 3.1), unidas a una estación de control, tal y como se muestra en la Figura 3.2.

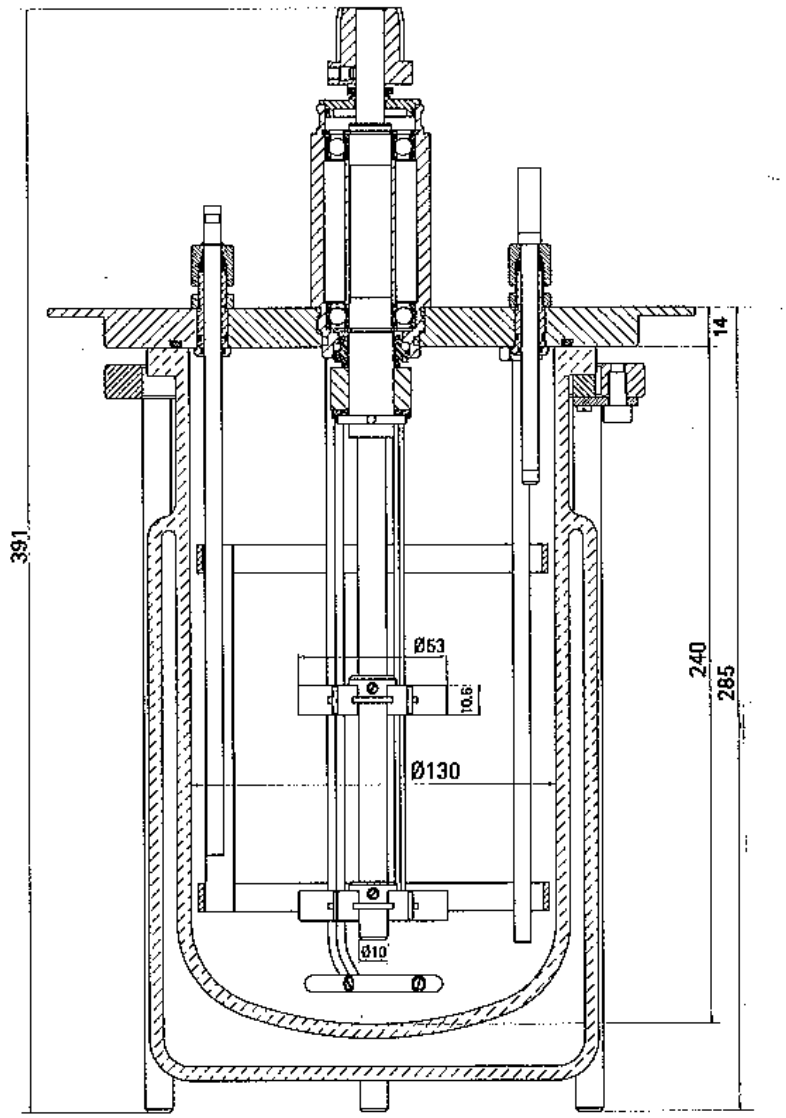

Figura 3.1. Esquema de cuba de 2L de Sartorius BBI Systems. Medidas en mm.

En esta unidad se registran las señales emitidas por las sondas introducidas en la cuba y, a su vez, controla las variables de operación mediante el software MFCS/WIN3. El control de temperatura (en un rango máximo de 0-100 ºC) se lleva a cabo mediante un sensor Pt-100 con un error de ± 0.1 ºC. La temperatura se regula mediante la circulación de agua desionizada por un circuito cerrado gracias al equipo de refrigeración y recirculación de agua FRIGOMIX® 1000, de Sartorius. Mediante este sistema también se realiza la circulación de agua a través del condensador de la cuba de fermentación. El pH se controla gracias a un sensor Hamilton, cuyo rango de medida es de 2 hasta 12 unidades de pH, y la introducción de disoluciones ácidas o básicas cuyo caudal es impulsado por bombas peristálticas. Para la agitación (0-2000 rpm ± 10 rpm) se emplea un motor de 180 W que actúa sobre una varilla de acero inoxidable con una turbina tipo Rushton de 6 palas planas, la velocidad es medida por un tacómetro. El aire es conducido hasta la cuba gracias a la acción de compresores. Posteriormente, el gas es filtrado (0,2 µm diámetro de poro) e insuflado a través de un difusor toroidal. Se emplea un controlador de flujo másico que permite la mezcla de dos gases. En todos los casos, el tipo de acción de control que se aplica es PID (Proporcional, Integrativa, Derivativa).

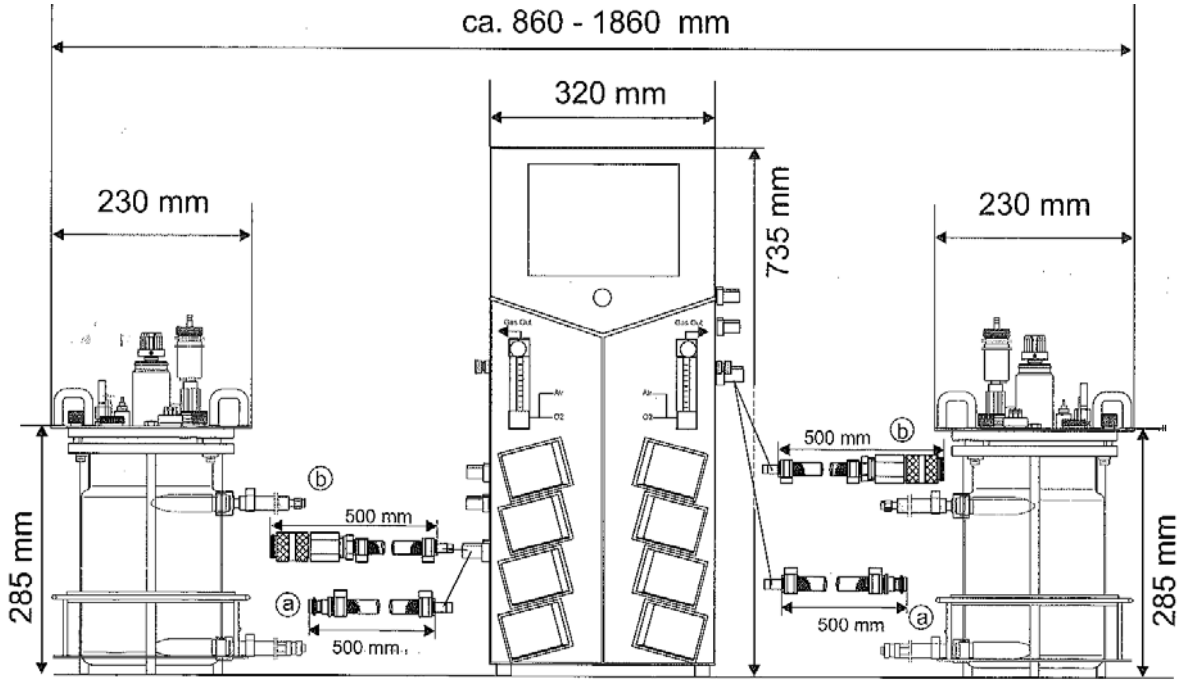

Figura 3.2. Esquema de la instalación de Sartorius BBI Systems, modelo BIOSTAT® B plus: estación de control conectada a dos cubas de 2 L.

3.2.3 Equipos de análisis

-Balanzas. En función de la magnitud de la pesada se ha empleado una balanza de precisión de Sartorius, modelo modelo Practum 1102-1S (capacidad máxima de 1100 g y precisión de ± 0,01 g) o una balanza analítica de MS204S de Mettler Toledo (capacidad máxima 220 g y precisión de ± 0,0001 g).

-Cromatógrafo de líquidos de alta resolución. La concentración de azúcares y ácidos de las muestras se determinaron mediante un cromatógrafo de líquidos Agilent, modelo Series 1100, controlado con el software Agilent ChemStation®. El equipo consta de: una cabina de disolvente, con botellas que contienen los distintos eluyentes; un desgasificador de vacío para la eliminación de burbujas de la fase móvil; una bomba cuaternaria que impulse un determinado caudal del eluyente; un inyector automático capaz de succionar un volumen de muestra programado e introducirlo en una válvula de seis vías para su mezcla con la fase móvil; el horno de columna termostatizado para control de la temperatura en la columna de separación de los componentes de la muestra; un detector de diodos con una lámpara de deuterio y una matriz de fotodiodos, para la obtención de un espectro de absorción UV-Vis (Ultravioleta-Visible); y un detector de índice de refracción con una celda de muestra y otra de referencia.

-Espectrofotómetro de absorción UV-Vis. La concentración de la biomasa se determinó mediante un espectrofotómetro de doble haz de Shimadzu, modelo UV 1603 con un intervalo de longitud de onda de 190-100 nm, ancho de banda espectral de 2 nm. Para las medidas en la región UV se emplea una lámpara de deuterio, mientras para la región visible e infrarrojo próximo se utiliza una lámpara halógena de tungsteno.

-pH-metro. Para control del pH de las fermentaciones en botella se recurrió a un medidor de CRISON, modelo Basic 20. Este equipo es capaz de medir valores comprendidos entre 2 y 16 unidades de pH con un error de ± 0,01.

-Transmisor y sonda de CO_2. Para determinar la concentración de CO_2 disuelto se utilizó un sensor InPro 5000(i) conectado a un transmisor multiparamétrico M400, siendo ambos dispositivos de la marca Mettler Toledo. En rango de medida es de 0-5000 g L^{-1} CO_2 disuelto (0-200 % de saturación, 0-2000 mbar), con una precisión de ± 1 dígito.

-Viscosímetro. Equipo de tipo caída de bola de Anton Paar, modelo AMNv, con un rango de medida de 0,3-1000 ± 0,001 mPa·s.

3.2.4 Equipos auxiliares

-Centrífuga. Equipo de SIGMA, modelo 4-16K, utilizado tanto en la separación celular del caldo de cultivo como en ciertos pasos del pretratamiento de los hidrolizados. Permite controlar la temperatura entre -20 y 70 ºC y consta de un rotor con una velocidad máxima de 9000 rpm para botes de 250 mL o un rotor capaz de alcanzar 15000 rpm para tubos de Eppendorf de 1.5 mL.

-Estufa de secado. Marca Selecta, modelo S-202, capaz de controlar la temperatura entre 40-200 ± 0,5 ºC.

-Manta calefactora. Marca Nahita, modelo 655 de 500 mL. Dispositivo utilizado para la preparación de hidrolizados. Temperatura máxima alcanzable de 370 ºC.

-Sistema de purificación de agua. Equipo de Younglin, aquaMAX^{TM}-Ultra 370, con una resistividad de 18. MΩ·cm, concentración de carbono orgánico total (Total Organic Carbon-TOC) de 5~10 ppb y un caudal máximo de 1,5 L min^{-1}.

-Microscopio óptico. Marca Zeiss, modelo Axioplan 2.

3.3 PROCEDIMIENTOS EXPERIMENTALES

3.3.1 Hidrólisis de residuos

Trozos de patata

Los trozos de patata se lavaron, pelaron y trituraron hasta convertirlos en puré. Posteriormente, se llevó a cabo una hidrólisis ácida. Se utilizó un matraz de fondo redondo de 0,5 L con condensador de reflujo. La temperatura del proceso se controló con una manta calefactora. En el matraz se vertieron 150 mL de HCl 1M y se calentó hasta alcanzar los 100 °C, momento en el que se vertieron 150 g de puré de patata. Las muestras se neutralizaron con KOH 5 M y se centrifugaron a 9000 rpm durante 10 minutos. El

sobrenadante del hidrolizado (HRP) fue filtrado a través de poros de 0,22 µm de diámetro para su esterilización. El protocolo de hidrólisis se basó en el procedimiento propuesto por Tasić et al. [140].

Bagazo de cerveza

El bagazo de cerveza fue secado a 40 ºC durante 48 h, molido y tamizado hasta alcanzar un tamaño de 0,75 mm. Posteriormente, se mezcló con agua en una proporción 1:6 m/v y se ajustó a pH 5,5 con H_3PO_4. Posteriormente, se llevó a cabo la hidrólisis enzimática en 3 etapas. La primera de ellas se realizó a 90 ºC y 180 rpm durante 1 h, adicionando 1 mL de Termamyl® SC por cada litro de la mezcla de agua y bagazo. La adición de esta α amilasa permite hidrolizar los enlaces glucosídicos α-1,4 en la amilosa y la amilopectina, de modo que, el almidón se descompone rápidamente en dextrinas solubles y oligosacáridos [141]. En la siguiente etapa se bajó la temperatura a 55 ºC y se procedió a la hidrólisis durante 1 h a 180 rpm gracias a la adición de 0,3 mL L^{-1} de Saczyme® Yield, 0,3 mL L^{-1} de FAN Boost™ y 0,3 mL L^{-1} de Ultraflo®XL. En la última etapa, se ajustó el pH a 5 (H_3PO_4) y se completó la hidrólisis durante 10 h a 180 rpm y 45 ºC con 15 mL L^{-1} de Celluclast 1.5L. La actividad enzimática clave de UltraFlo® MAX la proporciona una mezcla de endo-β-glucanasas que hidrolizan los enlaces (1,3) o (1,4) en los β-D-glucanos y xilanasa que hidroliza enlaces (1,4)-β-D-xilosídicos en xilanos, la acción de la endoproteasa FAN Boost™ permite lograr la hidrólisis de los enlaces péptidos internos y gracias a la glucoamilasa Saczyme® se consigue descomposición de dextrinas en azúcares simples [142–144]. Celluclast es un cóctel de endo- y exo-glucanasas que permite la escisión del polímero de celulosa en azúcares más pequeños y polisacáridos oligoméricos. El hidrolizado de bagazo de cerveza (HBC) resultante se centrifugó durante 20 min a 4 ºC y 8000 rpm. El sobrenadante se sometió a insuflación de aire durante 6 h a 50 °C para eliminación de volátiles y, finalmente, se purificó con carbón activado (CA). Todas las enzimas utilizadas fueron suministradas por Novozymes. Este protocolo está basado en el procedimiento publicado en el trabajo de Xu et al. [113].

Levadura de cerveza

La levadura de cerveza gastada se mezcló con agua en una proporción del 10% p/v y se ajustó a pH 6,8 mediante la adición de NaOH. La hidrólisis enzimática se realizó con el coctel de proteasas del producto comercial Alcalase® añadido en una proporción de 2g kg^{-1} de levadura de cerveza seca (la levadura de cerveza tenía una humedad del 43 % previamente determinada por pesada en seco) durante 12 h a 60 °C y 200 rpm. El

hidrolizado de levadura de cerveza gastada (HLCG) se centrifugó durante 15 min a 4 °C y 8000 rpm y se recogió el sobrenadante. Las enzimas fueron, de nuevo, suministradas por Novozymes. El procedimiento se basó en el propuesto por Jiang et al. [133].

3.3.2 Conservación del microorganismo

Para la preparación del stock del microorganismo se repartió el contenido completo del liofilizado de la ampolla (suministrada por GmbH) en una placa de agar con *Brain Heart Infusion* (BHI) y se incubó a 37 ºC durante 48 h. Posteriormente, las colonias fueron incubadas en botellas con 60 mL de *Tryptic Soy Broth* (TSB) a 37 ºC durante 24 h. Finalmente, el caldo de cultivo de mezcló con glicerol (1:1 v/v) y se almacenó en tubos tipo Eppendorf a -80 ºC,

3.3.3 Protocolo de reactivación y adaptación celular

Para la reactivación del microorganismo se inyecta el contenido de un Eppendorf de stock en una botella previamente autoclavada (121 ºC, 15 min) con 60 mL de TSB (2,5 g L^{-1} glucosa). El aire de esta botella ha sido previamente desplazado con N_2 a un caudal de 1 L min^{-1} durante dos minutos. Tras 24 h de cultivo a 37 ºC y 200 rpm, se procedió a la adaptación a la fuente de carbono, inoculando un 5 % (v/v) de biomasa en crecimiento en una botella en anaerobiosis con medio MPEL y 10 g L^{-1} del azúcar que se vaya a emplear posteriormente en la etapa de producción en reactor (las sales y la fuente de nitrógeno fueron esterilizadas por separado de la fuente de carbono). Tras una incubación de 24 h en las mismas condiciones que la etapa de reactivación, se inyecta un 5 % (v/v) de biomasa en otra botella igual que su precedente, pero cuya concentración de azúcar ha sido incrementada 10 g L^{-1}. Este procedimiento de adaptación mediante incrementos de 10 g L^{-1} de azúcar se repite hasta alcanzar la concentración de la fuente de carbono con la que se vaya a trabajar en la producción a escala de reactor.

En el caso de que posteriormente se fuera a realizar una producción a partir de residuos como fuente de carbono, es necesario añadir etapas de adaptación al hidrolizado, incrementando la relación hidrolizado/azúcar puro en sucesivos crecimientos hasta sustituir completamente el azúcar comercial. Para las producciones con sustitución de la fuente de nitrógeno por un el hidrolizado de un residuo no se precisa de una adaptación previa del microorganismo.

3.3.4 Experimentos de producción de ácido succínico

Para los experimentos de producción de producción de ácido succínico se precisa partir de un inóculo cuyas células se encuentren en un estado de crecimiento adecuado y cuya concentración esté controlada. Para mejorar la reproducibilidad de los experimentos se puede incluir una de etapa de preinóculo en botellas anaerobias con medio MPEL y la concentración de azúcar que se empleará en la etapa de producción. En ellas se inyecta un determinado volumen de caldo de cultivo (valor calculado tras medida de la concentración celular en un espectrofotómetro UV-Vis) adaptado a la fuente de carbono que permita iniciar el crecimiento del preinóculo con 0,05 g L^{-1} de biomasa. Tras su incubación a 37 ºC y 200 rpm, una vez alcanzada la etapa exponencial de crecimiento, se inyecta en la botella de inóculo (idéntica a la de preinóculo) la suficiente biomasa para comenzar el crecimiento con una concentración celular de 0,05 g L^{-1}. Las condiciones de operación en la etapa de producción fueron seleccionadas tras un estudio bibliográfico previo, seleccionando aquellas de preferencia por la mayoría de autores [70,97–100,102].

Botellas - Células en crecimiento

Las producciones en botella se llevan a cabo bajo las mismas condiciones que las etapas de preinóculo e inóculo: a 37 ºC, 200 rpm, medio MPEL y partiendo de una concentración inicial de biomasa de 0,05 g L^{-1}. Para tomar de muestras sin una retirada excesiva del caldo de cultiva que pueda interferir en el metabolismo celular, se procede a realizar producciones con varias botellas a un mismo tiempo, iniciando las fermentaciones con 12 h de desfase.

Reactor - Células en crecimiento

Las producciones en reactor con células en crecimiento se han desarrollado en reactores de 2 L, con un volumen de trabajo de 1 L. El reactor, con medio MPEL en su interior, fue autoclavado a 121 ºC durante 15 min antes de su utilización. La fuente de carbono fue autoclavada o filtrada (en caso de ser un hidrolizado) aparte e introducida una vez la cuba estuviera atemperada. En el caso de emplear un hidrolizado como fuente de nitrógeno, el reactor se autoclavó conteniendo únicamente las sales en su interior y la fuente de nitrógeno se añadió a posteriori. Las fermentaciones cursaron a 37 ºC, 300 rpm, pH 6,8 (controlado por bombeo automático de NaOH 5M) y un caudal de CO_2 de 0,1 vvm. Los experimentos partieron de una concentración de biomasa de 0,05 g L^{-1}.

Reactor - Células en *resting*

Los experimentos con células en *resting* a partir de células en suspensión se llevaron a cabo en reactores de 1 L con un volumen de trabajo de 0,5 L, mientras que aquellas producciones realizadas a partir de las células inmovilizadas en el *biofilm* se desarrollaron en los mismos reactores de crecimiento de celular, es decir, en cubas de 2 L con un volumen de trabajo de 1 L.

Tras 15 h, 30 h o 40 h de fermentación en el reactor con células en crecimiento, la biomasa suspendida fue separada del caldo de cultivo mediante centrifugación (9000 rpm, 5 min) y lavado con una disolución de K_2HPO_4 50 mM. Inmediatamente después, la biomasa se inoculó en un segundo reactor de 1 L de capacidad con un volumen de trabajo de 0,5 L, en el cual se llevó a cabo la producción con células en estado de *resting*. Esta segunda cuba tan solo contiene una disolución de K_2HPO_4 50 mM y el azúcar comercial. Se operó en las mismas condiciones que con las células en estado de crecimiento: 37 ºC, 300 rpm, pH 6,8 y 0,1 vvm de CO_2.

En aquellos casos en los que se había observado la aparición de *biofilm* en el reactor de crecimiento celular (30 h, 40 h de fermentación), se realizaron algunos experimentos empleando las células inmovilizadas del *biofilm*, en vez de las suspendidas en el caldo, como biomasa en estado de *resting*. Para ello, el caldo de cultivo fue drenado cuidadosamente a través del tubo toma-muestras, procurando no dañar el *biofilm* adherido a las paredes del reactor. Después, a través de ese mismo tubo, se rellenó el reactor con K_2HPO_4 50 mM y el azúcar comercial.

Reactor - Operaciones tipo *fed-batch* y *repeated batch*

Las producciones tipo *fed-batch* y *repeated batch* se llevaron a cabo tanto con células en crecimiento como con células en estado de resting. Se utilizaron reactores de 1 y 2 L de volumen total operando a la mitad de dicho volumen en ambos casos.

En el caso de que se realizaran fermentaciones con células en estado de crecimiento, entonces las cubas estaban rellenadas de medio MPEL, pudiendo sustituir, en función del experimento, la fuente de carbono o de nitrógeno por hidrolizados. Si los experimentos tenían lugar con células en estado de *resting*, las cubas contenían exclusivamente azúcar y K_2HPO_4.

En las operaciones tipo *fed-batch*, se llevaron a cabo tres etapas, alimentando una solución concentrada de azúcar al inicio de cada uno de ellas. En fermentaciones tipo

repeated-batch, al final de la primera y segunda etapa, la biomasa suspendida fue separada del caldo líquido por centrifugación (9000 rpm, 5 min) y posteriormente inoculada en el reactor de la siguiente etapa. En todos los casos se operó a 37 ºC, 300 rpm, pH 6,8 y 0,1 vvm de CO_2.

3.3.5 Métodos de análisis

La evolución a lo largo del tiempo de las concentraciones de biomasa, sustratos y productos fueron controladas mediante la toma de muestras y posterior análisis durante el transcurso de los experimentos. También se monitorizaron las variaciones de concentración de oxígeno y dióxido de carbono disueltos.

Análisis de la concentración de biomasa

Dado que, la cuantificación de la biomasa se llevó a cabo mediante espectrofotometría UV-Vis, fue preciso determinar previamente la constante de calibración de la biomasa en el equipo. Esto se consiguió mediante, por un lado, la realización de diluciones de un cultivo de *A. succinogenes* del cual, por otro lado, se determinó su peso seco. Gracias al peso seco se pudo conocer el valor de la concentración de biomasa del cultivo y, gracias a las medidas de las diluciones en el espectrofotómetro a 600 nm se pudo calcular la recta de calibrado que se muestra en la ecuación (3.1).

$$C_X = (0{,}846 \pm 0{,}01) \cdot Abs_{600\,nm} \qquad \text{(Ec. 3.1)}$$

Una vez determinada se puede proceder a la medida de la concentración de biomasa de los experimentos de producción. En aquellos casos en que la fermentación tuviera lugar en botella y, por tanto, en presencia de $NaHCO_3$, fue necesario neutralizar las muestras con HCl para evitar turbidez no asociada a crecimiento celular.

Análisis de la concentración de reactivos y productos

Tras centrifugar y filtrar las muestras (diámetro de poro = 0,2 µm), las concentraciones de azúcares y ácidos se determinaron mediante HPLC.

Los azúcares de los hidrolizados fueron cuantificados mediante una columna de plomo (8 %, 300 x 7,8 mm) BP-800 de Benson, utilizando como fase móvil 0,5 L min^{-1} de agua ultrapura. El horno mantuvo la columna a 80 ºC y RID operó a 55 ºC.

Los ácidos generados durante la fermentación fueron analizados con una columna de protones (8 %, 300 x 7,8 mm) BP-800 H de Benson. La fase móvil fue H_2SO_4 5 mM bombeado a un caudal de 0,5 L min^{-1}. De nuevo, se trabajó con una temperatura de 80 ºC en el horno y de 55 ºC en el RID.

3.3.6 Modelización cinética y método de ajuste

A partir de la evolución de las concentraciones de la biomasa, azúcares, ácido succínico y subproductos se han propuesto modelos cinético cuyos parámetros se han determinado a partir de los datos experimentales gracias al software informático Aspen Custom Modeler v11 (AspenTech, EE.UU). Para integrar las ecuaciones diferenciales y estimar los parámetros aplicando el método de mínimos cuadrados, se ha utilizado el método implícito de Euler acoplado a un algoritmo de regresión no lineal (NL2SOL). NL2SOL es una biblioteca FORTRAN77 que implementa un algoritmo adaptativo de mínimos cuadrados no lineales, desarrollado por John Dennis, David Gay y Roy Welsch [145].

Los parámetros estadísticos que se han estudiado para determinar la bondad de ajuste son los que se muestran en las ecuaciones (3.2-3.4). El valor F de Fisher (F) (Ec. (3.11)) es adecuado cuando es mayor que su tabulado al 95 % de confianza para que la hipótesis nula no pueda ser rechazada. Su valor refleja un ajuste más confiable a medida que aumenta la diferencia entre los valores de las varianzas del numerador y el denominador. El error de la raíz cuadrada media (Root Mean Square Error - RMSE) (Ec. (3.12)) muestra las diferencias entre los valores predichos por el modelo cinético propuesto y los valores experimentales utilizando la distancia euclidiana, por lo tanto, este parámetro debe ser lo más cercano a cero posible. La variación explicada (VE) (Ec. (3.13) mide la proporción en la que el modelo cinético explica la dispersión del conjunto de datos experimentales, por lo que la bondad de un modelo cinetico está relacionada con altos porcentajes de VE [146].

$$F = \frac{\sum_{i=1}^{N}\left(\frac{y_{i,calc}}{K}\right)^2}{\sum_{i=1}^{N}\left(\frac{SSR}{N-K}\right)} \qquad \text{(Ec. 3.2)}$$

$$RMSE = \sqrt{\frac{SSR}{N-K}} \quad \text{(Ec. 3.3)}$$

$$VE(\%) = 100\left(1 - \frac{\sum_{l=1}^{L} SSQ_l}{\sum_{l=1}^{L} SSQ_{mean_l}}\right) \quad \text{(Ec. 3.4)}$$

$y_{i,calc}$ son los valores calculados, K es el número de parámetros, SSR es la suma de residuos al cuadrado, N es el número de datos experimentales, SSQ_l es la suma de los residuos al cuadrado and SSQ_{meanl} es la suma al cuadrado de las desviaciones entre los valores experimentales y los valores medios con respecto a los valores calculados.

4. DISCUSIÓN INTEGRADORA

4 DISCUSIÓN INTEGRADORA

En este trabajo se ha abordado el estudio de la producción de ácido succínico mediante la acción de *A. succinogenes* empleando tanto glucosa como xilosa como fuentes de carbono. De forma esquemática, el trabajo realizado se recoge en la Figura 4.1, en la que se observan diversas etapas. En primer lugar, se ha puesto a punto un método experimental de adaptación del microorganismo a la fuente de carbono, así como la preparación de los inóculos a emplear en los estudios posteriores, en los que se ha considerado la influencia de distintas variables y condiciones de operación en el crecimiento celular y rendimiento de producción en reactor (concentración inicial de biomasa, uso de extracto de levadura, caudal de CO_2, velocidad de agitación). Asimismo, se ha comprobado la viabilidad de la producción de ácido succínico con *A. succinogenes* no solo empleando las células en crecimiento, sino también en estado de *resting*. Por otra parte, se ha estudiado la influencia de la forma de operación en el proceso, realizando operaciones en *batch, repeated batch* y *fed-batch*. Finalmente, los resultados obtenidos en estos estudios en los que se emplearon los azúcares puros, se extrapolaron al empleo de residuos alimentarios que, una vez sometidos a tratamientos de hidrólisis ácida o enzimática, fueron empleados como fuente de carbono o de nitrógeno. Se utilizaron residuos de patata y de bagazo de cerveza como fuente de carbono rica en glucosa y xilosa, respectivamente. En sustitución del extracto de levadura comercial, como fuente de nitrógeno, se recurrió a levadura de cerveza gastada y en vez de glucosa o xilosa como fuente de carbono, se utilizaron residuos de patata y de bagazo de cerveza, respectivamente. Tras comprobar la posibilidad de fermentar estos residuos en *batch*, se optimizó el proceso de producción con operaciones tipo *repeated batch* a partir de los residuos de patata y de levadura de cerveza gastada. Asimismo, se plantearon modelos cinéticos capaces de ajustar todos los resultados experimentales, tanto con células en estado de crecimiento como de *resting*, con distintos modos y condiciones de operación. Los modelos planteados son de tipo no segregado-no estructurado y son capaces de ajustarse a la evolución de las concentraciones del sustrato, productos, subproductos y biomasa a lo largo de la fermentación. Finalmente, se está llevando a cabo un estudio fenomenológico sobre la transferencia de dióxido de carbono, pero debido a la no disponibilidad de tiempo para comenzar los trámites de entrega de este documento, en este trabajo se incluye únicamente un estudio preliminar sobre la influencia de la transferencia de dióxido de carbono en el proceso de producción de ácido succínico.

El trabajo llevado a cabo en la presente Tesis Doctoral se ha recogido en las siguientes publicaciones (se incluyen completas en el ANEXO), a excepción del estudio de transferencia de CO_2, son las siguientes:

Publicación 1

Título: Modeling the Succinic Acid Bioprocess: A Review

Autores: Itziar A. Escanciano, Mateusz Wojtusik, Jesús Esteban, Miguel Ladero, y Victoria E. Santos

Revista: *Fermentation,* 8(8), 368 (2022)

https://doi.org/10.3390/fermentation8080368

Publicación 2

Título: On the succinic acid production from xylose by growing and resting cells of *Actinobacillus succinogenes*: a comparison

Autores: Itziar A. Escanciano, Miguel Ladero y Victoria E. Santos

Revista: *Biomass Conversion and Biorefinery,* 1-14 (2022)

https://doi.org/ 10.1007/s13399-022-02943-x

Publicación 3

Título: Development of a Simple and Robust Kinetic Model for the Production of Succinic Acid from Glucose Depending on Different Operating Conditions

Autores: Itziar A. Escanciano, Miguel Ladero, Victoria E. Santos y Ángeles Blanco

Revista: *Fermentation, 9(3), 222* (2023)

https://doi.org/10.3390/fermentation9030222

Publicación 4

Título: Study on the operational modes using both growing and resting cells for the succinic acid production from xylose. Kinetic modelling

Autores: Itziar A. Escanciano, Vanessa Ripoll, Miguel Ladero y Victoria E. Santos

Revista: *ACS Sustainable Chemistry & Engineering* (en revisión)

Publicación 5

Título: Bioproduction of succinic acid from potato waste: process development and kinetic modeling

Autores: Itziar A. Escanciano, Victoria E. Santos, Ángeles Blanco y Miguel Ladero

Revista: *Industrial Crops and Products* (en revisión)

Publicación 6

Título: Integral use of brewery wastes as carbon and nitrogen sources for the bioproduction of succinic acid

Autores: Itziar A. Escanciano, Ángeles Blanco, Victoria E. Santos y Miguel Ladero

Revista: *Bioresource Technology* (en revisión)

Publicación 7

Título: Succinic acid production by *Actinobacillus succinogenes* using acid and enzymatic hydrolysates of potato and beer wastes and repeated batch operation

Autores: Itziar A. Escanciano, Miguel Ladero, Ángeles Blanco y Victoria E. Santos

Revista: *Chemical Engineering Journal* (en revisión)

A continuación, se van a ir abordando las diferentes etapas del trabajo comentadas, relacionándolas con las publicaciones enumeradas.

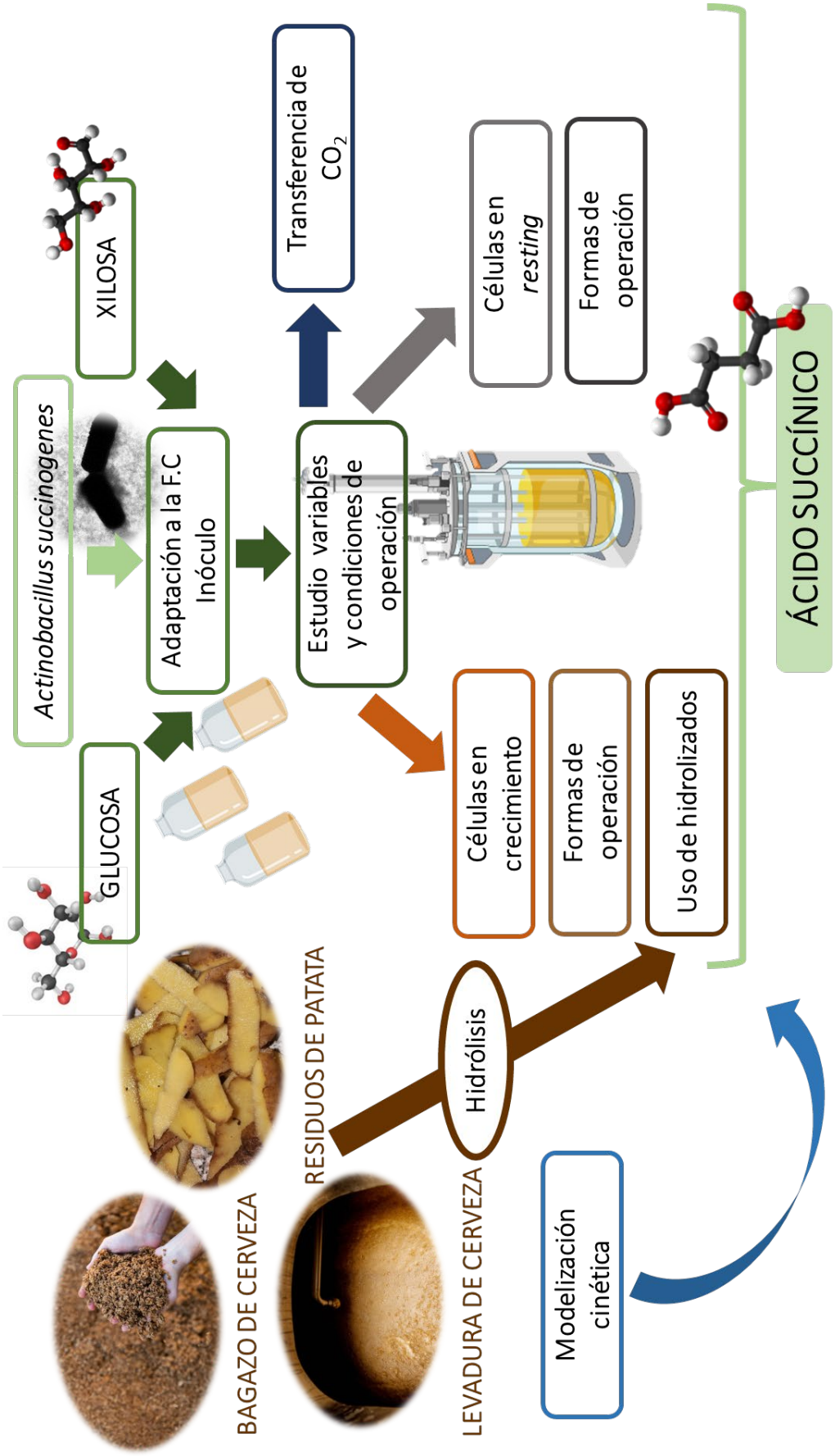

Figura 4.1. Esquema general del trabajo recogido en esta Memoria.

4.1 ADAPTACIÓN A LA FUENTE DE CARBONO Y PREPARACIÓN DEL INÓCULO.

Para poder estudiar la influencia de las variables, fenómenos o formas de operación comentados anteriormente, es necesario conseguir resultados experimentales reproducibles. Por ello, es de suma importancia prestar atención a esta primera etapa del estudio. Cuando las células se transfieren de un medio con una determinada cantidad de azúcar a otro con una fuente de carbono y/o concentración diferente, con frecuencia es necesario un período de adaptación que permita la activación de los mecanismos de las redes de reacciones implicadas. Durante las fases de latencia, los intermediarios necesarios deben acumularse en las combinaciones apropiadas y, durante los procesos de adaptación, se produce un crecimiento lento con la modificación del equilibrio enzimático real de la célula [147]. Por ello, se optó por iniciar un estudio sobre la influencia en la producción de ácido succínico de la realización una adaptación de *A. succinogenes* a la fuente de carbono, así como de la adición de una etapa de preinóculo. La mejora de la reproducibilidad de los resultados experimentales debido a la inclusión de este último paso ha sido ampliamente estudiado por este grupo de investigación con otros microorganismos [148–152]. Los resultados de este estudio se recogen en la **Publicación 2** "*On the succinic acid production from xylose by growing and resting cells of* Actinobacillus succinogenes*: a comparison*.", cuyos principales resultados se resumen a continuación.

Tal y como se detalló en el apartado 3.3.3, la adaptación celular se llevó a cabo tras la reactivación de las células congeladas mediante crecimientos sucesivos en botella con cantidades crecientes de la fuente de carbono. Tras esta etapa se consideró la posibilidad de realizar un crecimiento extra previo al inóculo, el preinóculo. Los resultados obtenidos de la evolución de la concentración de biomasa (C_X) en el tiempo en las etapas de preinóculo e inóculo llevadas a cabo en botella se muestran en la Figura 4.2. En la Tabla 4.1 se indica la numeración de estos experimentos y su estado adaptación y número de etapas de inóculo (preinóculo e inóculo o solamente una etapa).

En la Figura 4.2 se puede observar que la adaptación a la fuente de carbono y la realización de dos etapas (preinóculo – inóculo) permite aumentar la velocidad de producción de biomasa y su concentración final. Este hecho sugiere que, como es común en la práctica industrial, el inóculo debe crearse a través de más de una etapa, aumentando

la viabilidad del microorganismo de una etapa a la siguiente. Gracias a las sucesivas etapas de crecimiento se puede evitar una excesiva densidad celular al inicio del proceso, ya que la pequeña concentración de células viables se adapta fácilmente al medio de producción con suficiente espacio y nutrientes para proliferar, evitando la apoptosis o muerte celular programada [153].

Tabla 4.1. Experimentos realizados para el estudio del efecto de la adaptación y la etapa de preinóculo en el crecimiento en botella a partir de xilosa.

Exp.	**1**	**2**	**3**	**4**
Adaptación	No	Sí	No	Sí
Nª etapas inóculo	1	1	2	2

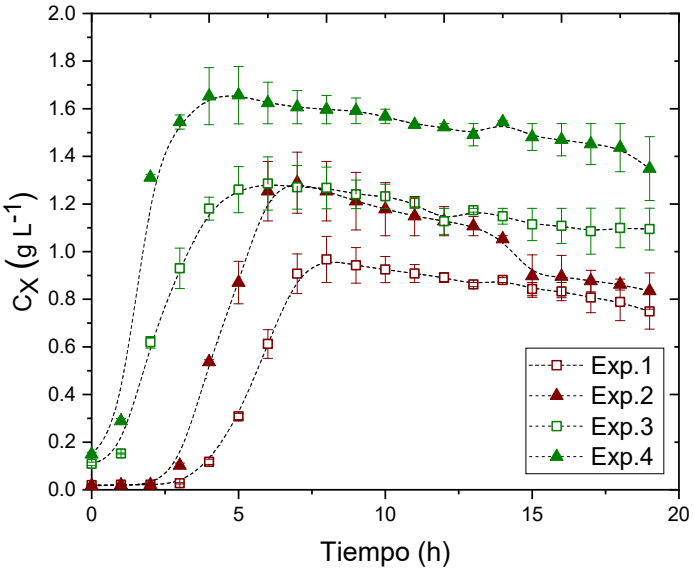

Figura 4.2. Efecto de la adaptación de las células a la presencia de xilosa en el medio de cultivo sobre el crecimiento de biomasa en las etapas de preinóculo e inóculo en botella.

Por otra parte, se observó la influencia de esta adaptación y metodología de preparación del inóculo en la producción de ácido succínico en reactor. La Figura 4.3 muestra el consumo de xilosa (C_{XIL}) y la producción de biomasa, ácido succínico (C_{SA}) y subproductos (C_{SP}), aplicando *lumping* a los ácidos acético (AA) y fórmico (FA), para cuatro experimentos en *batch* realizados bajo idénticas condiciones a partir de 20 g L^{-1} de xilosa. Las diferencias entre ellos radican en realizar (o no) una adaptación de la cepa a

la xilosa y en el número de etapas de inóculo (una o dos). En la Tabla 4.2 se presenta un resumen de los parámetros clave de fermentación obtenidos en ausencia y presencia de una adaptación progresiva a la xilosa y el uso de una o dos etapas de inóculo en los experimentos realizados para determinar su influencia en la producción de ácido succínico en biorreactor.

Tabla 4.2. Valores de parámetros de fermentación en *batch* a partir de xilosa en función de las etapas de adaptación y de preinóculo.

Exp.	**5**	**6**	**7**	**8**
Adaptación	**No**	**No**	**Sí**	**Sí**
Preinóculo	**No**	**Sí**	**No**	**Sí**
C_{SA} (g_{AS} L^{-1})	3,19	5,83	6,82	8,72
C_{SA} / C_{SP} (g_{SA} g_{SP}^{-1})	3,02	3,40	1,86	1,74
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,15	0,27	0,33	0,44
$Y_{SA/S.cons}$ (g_{SA} $g_{S.cons}^{-1}$)	0,78	0,79	0,33	0,44
$Y_{SA/X}$ (g_{SA} g_{X}^{-1})	1,35	2,37	2,24	2,80
P_{SA} (g_{SA} L^{-1} h^{-1})	0,11	0,22	0,30	0,36

En la Figura 4.3 se puede observar cómo la adaptación progresiva del microorganismo a la xilosa incrementa su velocidad de crecimiento y la concentración de biomasa en la fase estacionaria. Con dos etapas de preparación del inóculo (preinóculo + inóculo) antes de la etapa de producción, se produce un aumento de la biomasa, así como de la producción de ácido succínico y subproductos. Además, cuando se realiza la adaptación, la xilosa se consume totalmente al partir de una concentración relativamente baja (20 g L^{-1}). Por tanto, es posible plantear la hipótesis de que, en ausencia de una adaptación progresiva a la xilosa, los pasos metabólicos que conducen al ácido succínico están desactivados o fuertemente inhibidos. En general, estos fenómenos también afectan los rendimientos de los subproductos, cuya concentración final disminuiría de 5 g L^{-1} (Exp.8), con adaptación y etapa de preinóculo, hasta a 1 g L^{-1} (Exp.5), en ausencia de ambas etapas. Sin embargo, aunque la adición de estos pasos conduce a un incremento del rendimiento del ácido succínico de hasta un 173 % (3,19 g g^{-1} - Exp.5, 8,72 g g^{-1} - Exp.8), la selectividad de la fermentación resulta perjudicada, de hecho, la relación entre las concentraciones de succínico y subproductos disminuye desde 3,02 g g^{-1} en el Exp.5 hasta 1,74 g g^{-1} en el Exp.8 como se recoge en la Tabla 4.2.

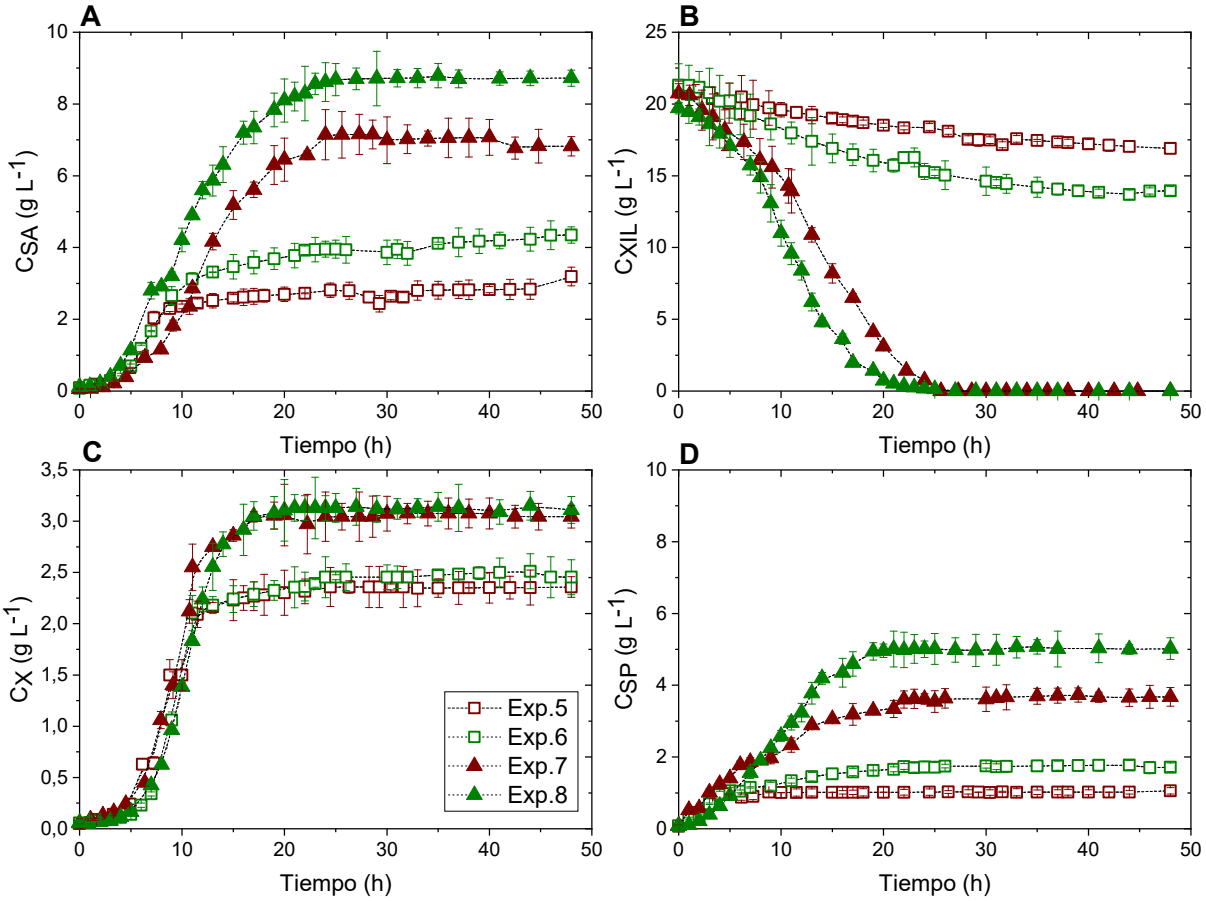

Figura 4.3. Efecto de la etapa de la adaptación de las células a la presencia de xilosa y la realización de un preinóculo sobre la producción de SA (A), consumo de xilosa (B), crecimiento de biomasa (C) y generación de subproductos (D) durante la fermentación con células en crecimiento en biorreactor.

4.2 ESTUDIO DE LA INFLUENCIA DE LAS VARIABLES Y CONDICIONES DE OPERACIÓN

En la bibliografía relativa a la bioproducción de ácido succínico se pueden encontrar trabajos centrados en el estudio de la influencia de determinadas variables en el proceso de fermentación, como la concentración de la fuente de carbono, de nitrógeno o de CO_2. Sin embargo, en algunos casos, no se ha conseguido un consenso entre las conclusiones de los distintos autores. Por ejemplo, en los estudios de la concentración óptima de glucosa, no sólo no se ha logrado una resolución en cuanto a la cantidad apropiada para incrementar en rendimiento de succínico, sino que ni siquiera se ha coincidido en las observaciones del comportamiento de la biomasa y la selectividad del

proceso en función de la cantidad de esta fuente de carbono [70,99,121]. Por ello, con el objetivo de optimizar el rendimiento y productividad del proceso, se realizó un estudio exhaustivo de la influencia que ejercen diversas variables clave ya estudiadas en bibliografía, como el caudal de CO_2, la concentración de extracto de levadura (C_{EL}) y la concentración de la fuente de carbono (C_S), así como variables en las que apenas se encuentran trabajos en la literatura, como la concentración inicial de biomasa (C_X) y la velocidad de agitación. Estos experimentos se realizaron en reactores de 2 L en operación tipo *batch*, produciendo ácido succínico a partir de glucosa mediante la acción de *A. succinogenes*. En la Tabla 4.3 se muestran los resultados para los catorce experimentos llevados a cabo en este estudio de los parámetros de fermentación como la concentración de ácido succínico, el rendimiento con respecto a la concentración inicial de la fuente de carbono, la productividad, y la selectividad de las fermentaciones realizadas bajo las distintas condiciones de operación mencionadas. En la **Publicación 3** "*Development of a simple and robust kinetic model for the production of succinic acid from glucose depending on different operating conditions*" y la **Publicación 5** "*Bioproduction of succinic acid from potato waste: process development and kinetic modeling*" se puede encontrar información más detallada de estos experimentos junto a una exhaustiva revisión bibliográfica del estado del arte del estudio de las condiciones de operación en la producción de ácido succínico, así como su discusión con relación a los resultados obtenidos en el presente estudio.

En la Tabla 4.3 la numeración de los 14 experimentos realizados comienza en el número 9, continuando con la numeración del apartado anterior. En los experimentos 9, 10 y 11, se estudió el efecto de la **concentración inicial de la biomasa** en la producción de ácido succínico. Tal y como se puede apreciar en la Tabla 4.3, este parámetro afecta principalmente a la productividad del proceso. Se observa que, a una concentración inicial de 0,075 g L^{-1}, valor intermedio de los estudiados, la productividad del ácido succínico alcanzaba un valor máximo de 0,96 g L^{-1} h^{-1}. Sin embargo, si se aumentaba aún más la concentración celular hasta 0,1 g L^{-1}, la productividad se reducía en un 21 %. Además, se ha observado un ligero aumento de la selectividad con cantidades iniciales más altas de biomasa en el reactor. Esta tendencia de la selectividad probablemente se encuentre asociada a posibles variaciones en el metabolismo. Para aclarar este punto, sería necesario realizar un análisis metabólico en fermentaciones realizadas a partir diferentes condiciones de tamaño de inóculo, como hicieron Din et al. [154] con *Saccharomyces*

cerevisiae, observando grandes cambios en los intermediarios del metabolismo de la glucosa, aminoácidos, así como metabolitos relacionados con la estructura de la membrana celular.

En los experimentos 9, 12 y 13 se estudia la influencia del **caudal de CO_2,** empleando valores de 0,1 L min^{-1} a 1 L min^{-1}, intervalo de valores representativos de los que se pueden encontrar en la bibliografía sobre producción de ácido succínico [70,98,100,119,121,123,155–159]. En este estudio no se han observado variaciones significativas en rendimiento, productividad o generación de subproductos en el rango de caudales empleado. Teniendo en cuenta el trabajo de Xi et al. [160] y Zou et al. [107], quienes logran ver diferencias en la producción de ácido succínico trabajando con mezclas de N_2 y CO_2 hasta llegar a la saturación de este último gas, se concluye que, al igual que en el presente trabajo, las fermentaciones con exceso de CO_2 no favorecen la desviación del metabolismo hacia la generación de ácido succínico en detrimento de otros metabolitos, llegando a la conclusión de que la mayoría de los estudios sobre producción de ácido succínico por *A. succinogenes* publicados hasta la fecha se están realizando en condiciones que implican mayores costes económicos y no ofrecen ninguna ventaja adicional.

En los experimentos 9, 14, 15 y 16 se estudió el efecto del incremento de la **velocidad de agitación** entre 150 rpm y 300 rpm, rango de valores típicamente usados según bibliografía [70,97,98,100,119,121,155,157–159,161]. En la Tabla 4.2 se advierte una mejora considerable en rendimiento y productividad a altos valores de agitación, alcanzando los mejores resultados a 250 rpm. Esta condición de operación es uno de los factores con mayor impacto en la transferencia de gases en medios líquidos, disminuyendo el tiempo de mezclado y mejorando las tasas de transferencia de masa y calor [162,163]. Por lo tanto, esto podría apuntar a que los valores de agitación de 150 rpm y 200 rpm podrían ser insuficientes para lograr una adecuada transferencia de CO_2 en el caldo de cultivo. Teniendo en cuenta este fenómeno, se podría inferir que, a mayor velocidad de agitación, mayor generación de ácido succínico; sin embargo, fuerzas de cizallamiento excesivas pueden conducir al daño de la celda y, como consecuencia, a la reducción del rendimiento del proceso [164], como parece estar ocurriendo en el experimento llevado a cabo a 300 rpm, en el que parece quedar patente el efecto de un posible exceso de estrés hidrodinámico.

Tabla 4.3. Valores de parámetros de fermentación en *batch* a partir de glucosa bajo diferentes condiciones de operación estudiadas.

Exp.	Tipo operación	C_X (g L^{-1})	Agitación (rpm)	Caudal CO_2 (L min^{-1})	C_{EL} (g L^{-1})	C_G (g L^{-1})	C_{SA} (g L^{-1})	$Y_{SA/S.0}$ (g g^{-1})	P_{SA} (g L^{-1} h^{-1})	S_{SA} (g g^{-1})
9	*Batch*	0,05	300	0.1	10	40	27,4	0,68	0,83	0,62
10	*Batch*	0,075	300	0.1	10	40	28,5	0,71	0,96	0,64
11	*Batch*	0,1	300	0.1	10	40	28,3	0,70	0,76	0,66
12	*Batch*	0,05	300	0.5	10	40	27,6	0,69	0,84	0,63
13	*Batch*	0,05	300	1	10	40	26,1	0,65	0,81	0,63
14	*Batch*	0,05	150	0.1	10	40	23,6	0,59	0,72	0,61
15	*Batch*	0,05	200	0.1	10	40	26,4	0,66	0,78	0,62
16	*Batch*	0,05	250	0.1	10	40	28,5	0,71	0,84	0,62
17	*Batch*	0,05	300	0.1	2,5	40	23,8	0,59	0,48	0,68
18	*Batch*	0,05	300	0.1	5	40	26,8	0,66	0,53	0,66
19	*Batch*	0,05	300	0.1	7,5	40	28,9	0,72	0,58	0,64
20	*Batch*	0,05	300	0.1	10	20	15,7	0,72	0,98	0,52
21	*Batch*	0,05	300	0.1	10	30	21,1	0,71	0,96	0,58
22	*Batch*	0,05	300	0.1	10	50	34,0	0,67	0,71	0,66

Hasta el momento, los estudios centrados en la **fuente de nitrógeno** son escasos y no se ha explorado a fondo el impacto que esta variable puede tener en la producción [105,126,165]. En los experimentos 9, 17, 18 y 19 se compara el efecto de la variación de la concentración inicial de la fuente de nitrógeno, es decir, del extracto de levadura (EL). A medida que la concentración de EL aumentó de 2,5 g L^{-1} a 10 g L^{-1}, también lo hizo la productividad del ácido succínico, aunque en perjuicio de la selectividad, que disminuyó de un 68 % a un 62 %. Sin embargo, el rendimiento de ácido succínico con respecto a la concentración inicial de la fuente de carbono alcanzó su máximo en las fermentaciones realizadas con 7,5 g L^{-1} de EL.

En los experimentos 9, 20, 21 y 22, realizados a diversas **concentraciones de glucosa** comprendidas entre 20 g L^{-1} y 50 g L^{-1}, se determinó que, concentraciones bajas de la fuente de carbono fueron favorables tanto para el rendimiento como para la productividad del ácido succínico, con valores de 0,72 g g^{-1} y 0,98 g L^{-1} h^{-1} para los experimentos a 20 g L^{-1} de sustrato y 0,68 g g^{-1} y 0,71 g L^{-1} h^{-1} al trabajar con 50 g L^{-1} de glucosa. Se observó una tendencia creciente de la selectividad con la concentración inicial de glucosa, desde 0,5 g g^{-1} a 20 g L^{-1}, hasta 0,7 g g^{-1} a 50 g L^{-1} de sustrato.

4.3 ESTUDIO DE LA PRODUCCIÓN DE ÁCIDO SUCCÍNICO CON *RESTING CELLS*

Las biotransformaciones catalizadas por células pueden tener lugar cuando las células están en estado de crecimiento, pero también cuando las células se encuentran en estado de reposo o *resting*. Las células en estado de *resting cells* no experimentan crecimiento, sin embargo, son metabólicamente muy activas, esta situación permite separar el proceso en dos etapas: una etapa de producción del biocatalizador y otra de biotransformación. La centrifugación permite separar la biomasa en estado de crecimiento obtenida en la primera etapa, consiguiendo un biocatalizador que posteriormente se utiliza en la etapa de producción, suspendido en un medio compuesto exclusivamente por la fuente de carbono y una solución tampón que permite el mantenimiento de las condiciones de presión osmótica. En general, el propósito de este tipo de operación catalizada por células en estado de *resting*, es dirigir el metabolismo hacia el producto de interés minimizando la generación de subproductos [152,166–168], lo que resulta especialmente conveniente en la producción de ácido succínico, pues las

técnicas de purificación suponen uno de los principales costes en la producción de este compuesto [169]. Otra de las ventajas de operar con *resting cells* es la posibilidad de optimizar independientemente las etapas de crecimiento y producción, pudiendo evitar fenómenos inhibitorios debidos a sustratos y productos asociados al crecimiento celular [152,166,168]. En este trabajo se quiso comprobar la viabilidad de producir succínico con *A. succinogenes* en estado de *resting*, determinando, además, el estado óptimo de la biomasa en crecimiento que se emplea como inóculo, determinando el grado de adaptación y número de etapas de inóculos necesarias, edad, estado celular (células libres o inmovilizadas) y su medio de cultivo, que se van a resumir en los siguientes subapartados. Los resultados que se presentan en este apartado y su discusión pormenorizada se pueden encontrar en la **Publicación 2** "*On the succinic acid production from xylose by growing and resting cells of Actinobacillus succinogenes: a comparison*." y la **Publicación 4** "*Study on the operational modes using both growing and resting cells for the succinic acid production from xylose. Kinetic modelling*".

4.3.1 La adaptación celular para la viabilidad de la producción con células en estado de *resting*.

A. succinogenes es capaz de producir ácido succínico después de que se detenga el crecimiento celular. De hecho, según Der Werf et al. [170], la tasa de producción de ácido succínico permanece constante antes y después del final de la fase de crecimiento exponencial. Este es un punto clave a la hora de utilizar bacterias en estado de reposo como biocatalizador.

En este estudio, la producción de ácido succínico se ha llevado a cabo en reactores en los que se detiene la reproducción celular por ausencia de una fuente de nitrógeno. Estas células se inoculan en una concentración elevada, procedentes de reactores cuyo medio de cultivo contiene una fuente de nitrógeno y las sales necesarias para el crecimiento del microorganismo. La Figura 4.4 muestra la evolución de las especies involucradas en una fermentación tipo *batch* con células en estado de *resting* a partir de 20 g L^{-1} de xilosa en función de si previamente las células han sido adaptadas a la fuente de carbono y han crecido en una o dos etapas de inóculo. En la Tabla 4.4 se muestran los resultados correspondientes de concentración de producto, rendimiento y productividad alcanzados de los cuatro experimentos realizados para este estudio.

Tabla 4.4. Valores de parámetros de fermentación en la producción de ácido succínico en *batch* con *resting cells* a partir de xilosa en función de las etapas de adaptación y de preinóculo.

Exp.	**23**	**24**	**25**	**26**
Adaptación	**No**	**No**	**Sí**	**Sí**
Preinóculo	**No**	**Sí**	**No**	**Sí**
C_{SA} (g_{SA} L^{-1})	0,28	0,80	2,30	8,51
C_{SA} / C_{SP} (g_{SA} g_{SP}^{-1})	-	5,70	2,63	2,97
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,01	0,04	0,11	0,43
$Y_{SA/S.cons}$ (g_{SA} $g_{S.cons}^{-1}$)	1,14	0,53	0,65	0,81
$Y_{SA/X}$ (g_{SA} g_{X}^{-1})	0,08	0,24	0,56	2,03
P_{SA} (g_{SA} L^{-1} h^{-1})	0,01	0,04	0,12	0,18

Aunque, tal y como se mostró en el apartado 4.1 (con su operación equivalente con células en estado de crecimiento), la producción de ácido succínico con células en estado de crecimiento resulta favorecida adaptando el microorganismo al azúcar y añadiendo una etapa de preinóculo, se puede observar en la Figura 4.4 que, en el caso de utilizar células en estado de *resting*, estas etapas son imprescindibles. De hecho, con *resting cells*, las concentraciones finales de ácido succínico son 30 veces mayores adaptando la bacteria a xilosa y agregando una etapa de preinóculo, mientras que con células en crecimiento este incremento en la producción fue sólo 2,7 veces mayor.

Por tanto, cuando se utilizan células en estado de *resting*, el empleo de dos etapas de inóculo consecutivas conduce a un microorganismo más productivo. Sin embargo, una adaptación progresiva de la cepa a la xilosa es aún más influyente en el resultado del proceso [147]. Como estas células están sometidas a un gran estrés hidrodinámico debido al proceso de centrifugado y lavado, es evidente que requieren un proceso previo de refuerzo para no desactivarse en ausencia de una fuente de nitrógeno. De hecho, la producción de ácido succínico sin adaptación apenas llega a 1 g L^{-1}, deteniéndose inmediatamente el flujo metabólico. En el caso de realizar una etapa de adaptación, pero no de preinóculo, la tendencia de crecimiento en los primeros tiempos (hasta 6 h) es similar al caso en el que se agrega una etapa más de inóculo. Sin embargo, a partir de este momento sufre una desactivación, impidiendo que la concentración de ácido succínico supere los 2,3 g L^{-1}. Similar evolución se observa en la generación de subproductos, estabilizándose el valor en 0,8 g L^{-1}.

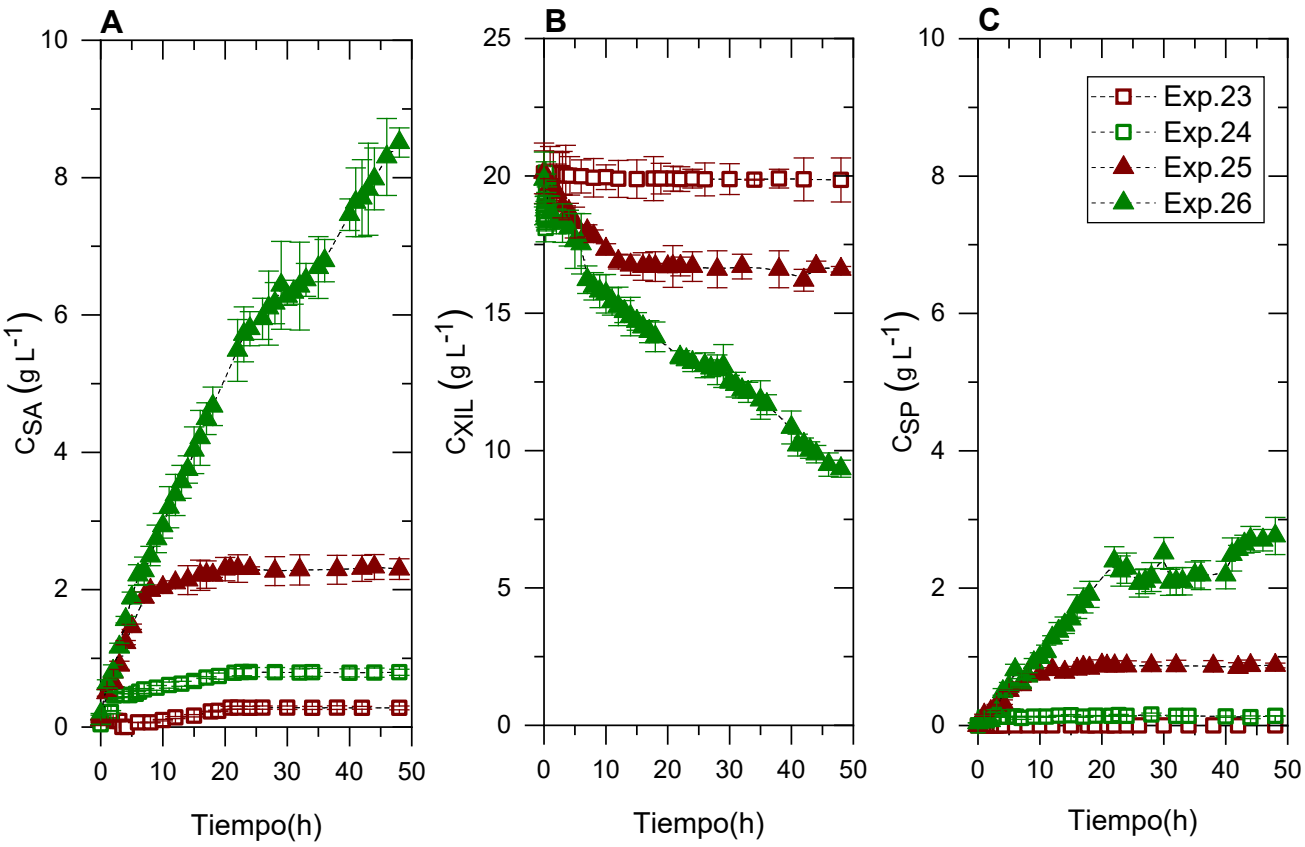

Figura 4.4. Efecto de la etapa de adaptación y preinóculo sobre la producción en *batch* de ácido succínico (A), consumo de xilosa (B), crecimiento de biomasa (C) y generación de subproductos (D) durante la fermentación con células en estado de *resting* en biorreactor a partir de xilosa.

Dependiendo del número de etapas de acondicionamiento del cultivo incluidas en el proceso, los rendimientos aumentan desde prácticamente 0 % hasta 43 % y las productividades desde 0,01 g L^{-1} h^{-1} hasta 0,18 g L^{-1} h^{-1} (Tabla 4.4). De hecho, en un proceso con etapa de adaptación y preinóculo, el rendimiento máximo alcanzado en ausencia de nitrógeno a las 48 h es muy similar al obtenido con células en estado de crecimiento (0,44 g g^{-1}, ver Tabla 4.2). Aunque la velocidad de producción con *resting cells* es menor que la de células en crecimiento, a las 48 h la producción sigue aumentando, quedando un remanente de 9,3 g L^{-1} de xilosa en el caldo de cultivo. Por tanto, se puede deducir que, a tiempos más avanzados y/o mayor concentración de biomasa inicial, el rendimiento podría llegar a alcanzar valores más elevados. Sin embargo, en la fermentación con células en crecimiento, a las 24 h, la xilosa ya se ha consumido por completo, alcanzando el máximo rendimiento del proceso, sin posibilidad de aumentar este valor a excepción de que se realizara un cambio a otro modo de

operación. Por otro lado, las vías de producción de los ácidos acético y fórmico parecen inactivarse alrededor de las 10 h en el caso de operar con células en estado de *resting* obtenidas mediante un proceso con etapa de adaptación y preinóculo mientras que la velocidad de generación de ácido succínico se mantiene constante. A partir de ese momento, el consumo de xilosa se dirige casi exclusivamente a la generación de ácido succínico, lo que permitiría alcanzar rendimientos muy superiores a tiempos más avanzados.

4.3.2 Influencia de la edad y estado celular (células libres o inmovilizadas en *biofilm*) en la producción con *resting cells*

A. succinogenes es un microorganismo capaz de generar *biofilm*, de modo que las células quedan inmersas en una matriz de producción propia de sustancias poliméricas extracelulares en forma de gel [137,156]. Esto tiende a ocurrir a tiempos de operación prolongados, adhiriéndose a las superficies de apoyo. Debido a esto, se quiso determinar el nivel de actividad de estas células inmovilizadas en el *biofilm* en ausencia de una fuente de nitrógeno y comparar su capacidad productiva con experimentos equivalentes con células en estado de suspensión.

En los reactores donde crecen las bacterias en presencia de una fuente de nitrógeno para ser empleadas como inóculo células en estado de *resting*, a las 15 h de fermentación todavía no se ha generado *biofilm*, de modo que solo hay células en suspensión. Sin embargo, a las 30 h se comienza a generar *biofilm*, encontrándose tan solo una pequeña cantidad adherida a las paredes del reactor. A las 40 h de fermentación, la cantidad de este *biofilm* ha aumentado considerablemente y no parece incrementar más su volumen. Teniendo esto en consideración, se realizaron experimentos con *resting cells*, empleando como inóculo aquellas bacterias procedentes del reactor de crecimiento celular a las 15 h de fermentación (libres) y, a tiempos más largos de fermentación (30 h y 40 h), a partir tanto de las células libres como de las células inmovilizadas en el *biofilm* adherido a las paredes del reactor (Figura 4.5). Se realizaron un total de cinco experimentos, cuyos resultados experimentales de las evoluciones de la concentración del sustrato, ácido succínico y subproductos se muestra en la Figura 4.6. Asimismo, los valores de los parámetros de fermentación se recogen en la Tabla 4.5.

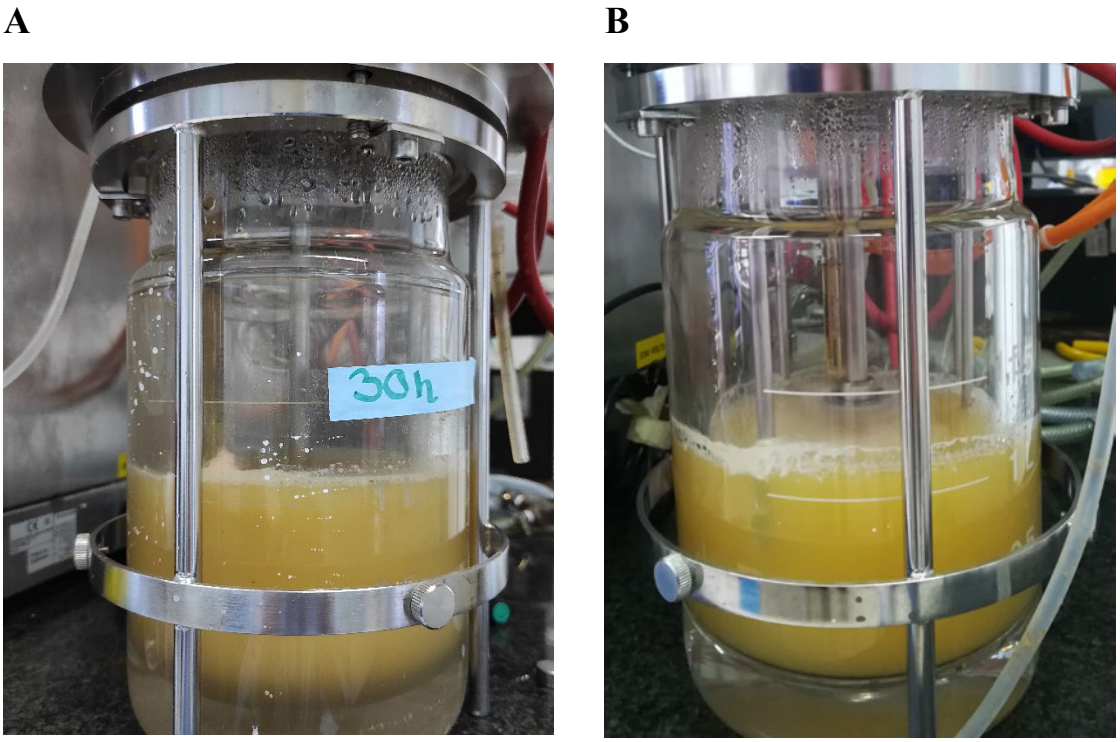

Figura 4.5. Formación de *biofilm* en el reactor de crecimiento celular a las 30 h (A) y a las 40 h (B)

Tabla 4.5. Valores de parámetros de fermentación en la producción en *batch* de ácido succínico con células en *resting* según la edad y el estado celular a partir de xilosa.

Exp.	**26**	**27**	**28**	**29**	**30**
Tiempo crecimiento (h)	**15**	**30**	**30**	**40**	**40**
Estado celular	**Libres**	**Libres**	***Biofilm***	**Libres**	***Biofilm***
C_{SA} (g_{SA} L^{-1})	8,51	3,36	0,29	0,12	3,28
C_{SA} / C_{SP} (g_{SA} g_{SP}^{-1})	2,97	2,34	1,02	1,57	0,50
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,43	0,17	0,01	0,01	0,17
$Y_{SA/S.cons}$ (g_{SA} $g_{S.cons}^{-1}$)	0,81	0,72	0,62	1,11	0,36
$Y_{SA/X}$ (g_{SA} g_{X}^{-1})	2,03	0,80	3,96	0,03	21,9
P_{SA} (g_{SA} L^{-1} h^{-1})	4,20	4,14	0,07	3,78	0,15
C_{SA} (g_{SA} L^{-1})	0,18	0,07	0,02	0,01	0,07

En la Tabla 4.5 se puede observar que los mayores rendimientos y productividades de ácido succínico se alcanzaron a partir de células libres tras 15 h de crecimiento (0,43 g g^{-1}, 0,18 g L^{-1} h^{-1}). Las producciones llevadas a cabo a partir de células libres a las 30 h de fermentación y a partir de células inmovilizadas a las 40 h de operación obtuvieron resultados idénticos (0,17 g g^{-1} y 0,07 g L^{-1} h^{-1}). Cabe señalar que, en las tres fermentaciones citadas, la velocidad de producción de ácido succínico se mantuvo prácticamente constante en el tiempo, con una cantidad considerable de xilosa aún en el

caldo a las 48 h, lo que sugiere que, a diferencia de aquellas fermentaciones realizadas en presencia de una fuente de nitrógeno, es decir, con células en estado de crecimiento (ver Figura 4.2), por lo que a tiempos más avanzados de la fermentación empleando células en estado de *resting* cells se podrían haber logrado mayores rendimientos. Sin embargo, en el caso de operar a partir de células inmovilizadas y células libres después de 30 h y 40 h de fermentación respectivamente, la producción de ácido succínico es muy baja, prácticamente despreciable.

Cabe destacar que, la concentración celular presente en el *biofilm* a las 40 h (determinada en peso seco) es 28 veces menor que la de la fermentación realizada a partir de células libres a las 15 h. Por lo tanto, los rendimientos de ácido succínico en relación a la biomasa serían de 21,9 g g^{-1} y 2,03 g g^{-1} respectivamente. Esto indica que la actividad metabólica de las células inmovilizadas es superior a las que se encuentran suspendidas en el caldo. Mokwatlo et al. [171] ya señalaron las ventajas de operar con *biofilm*. La matriz de sustancias poliméricas extracelulares le confiere muchos atributos beneficiosos, como una actividad sostenida a largo plazo y una mejor tolerancia a ambientes que de otro modo serían tóxicos. Además, las bacterias del *biofilm* constituyen una comunidad funcional coordinada más eficiente que las células planctónicas flotantes, lo cual es posible gracias a la cooperatividad fisiológica de las células bacterianas. El *biofilm* está protegido de la desecación, otorga a las células una mayor tolerancia frente a los agentes antimicrobianos y actúa como absorbente de nutrientes en la fase líquida.

En resumen, el modo de vida del *biofilm* proporciona a las células bacterianas características que son superiores a las del modo de vida en suspensión [137,171]. Sin embargo, en la fermentación, la alta densidad celular se puede definir como un requisito para mejorar la productividad volumétrica y, por tanto, reducir los gastos asociados a la producción. Por tanto, se requeriría de una estrategia de retención celular en la que las células se separen del caldo de fermentación y se concentren en el fermentador [104].

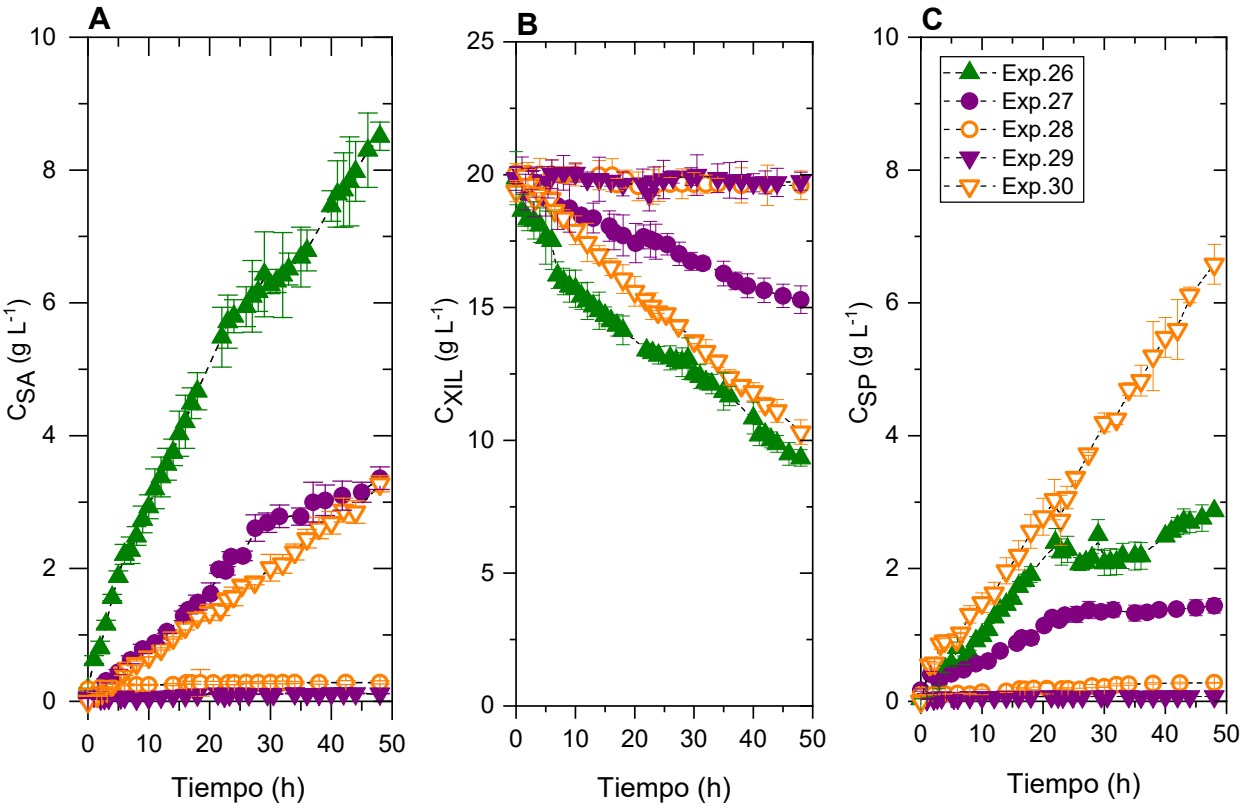

Figura 4.6. Influencia de la edad y estado celular de las *resting cells* en la producción en *batch* de ácido succínico (A), consumo de xilosa (B), generación de subproductos (C).

4.3.3 Influencia de la composición del medio empleando células en crecimiento y células en *resting*.

En todos los estudios sobre el empleo de células en estado de *resting cells* expuestos hasta este punto se ha empleado TSB, un medio complejo comercial, para el crecimiento celular previo a la etapa de producción con las células en estado de *resting*. Se había seleccionado este medio debido a su riqueza de nutrientes, considerado en bibliografía como un medio de predilección para favorecer el crecimiento celular [104,119,172]. Sin embargo, con el objetivo de abaratar el proceso, se quiso comprobar la viabilidad de llevar a cabo el crecimiento celular, previo a la operación con células en *resting*, con un medio de menor coste, MPEL. Este medio es el empleado en los estudios de la producción con células en estado de crecimiento en todos los demás experimentos presentados en esta tesis doctoral. En la Tabla 4.6, se muestra la comparación de la

producción con células en crecimiento y en *resting* con medios TSB y MPEL empleando como fuente de carbono 20 g L^{-1} de xilosa.

Tabla 4.6. Valores de parámetros de fermentación en la producción en *batch* de ácido succínico con células en crecimiento y en *resting* y dos medios de crecimiento diferentes a partir de xilosa.

Exp.	**8**	**31**	**26**	**32**
Biocatalizador	**Células en crecimiento**		***Resting cells***	
Medio de crecimiento	TSB	MPEL	TSB	MPEL
C_{SA} (g_{SA} L^{-1})	8,72	11,7	8,51	12,5
S_{SA} (g_{SA} g_{SA+SP}^{-1})	0,64	0,55	0,75	0,68
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,44	0,55	0,43	0,65
$Y_{SA/Scons}$ (g_{SA} $g_{S.cons}^{-1}$)	0,44	0,55	0,81	0,65
$Y_{SA/X}$ (g_{SA} g_X^{-1})	2,80	2,74	2,03	2,91
P_{SA} (g_{SA} L^{-1} h^{-1})	0,36	0,25	0,18	0,43
$P_{SA/X}$ (g_{SA} g_X^{-1} h^{-1})	0,12	0,06	0,04	0,10

En la citada Tabla 4.6 se puede observar que, en cuanto a la concentración del producto, el medio MPEL proporciona una mejor producción de SA, alcanzando altos valores de rendimiento de este ácido respecto a la fuente de carbono (0,55 g g^{-1} para estado de crecimiento y 0,65 g g^{-1} para estado de *resting*). Sin embargo, en ambos casos se aprecia un aumento de la generación de subproductos (ácido acético y ácido fórmico), hecho que se refleja en una ligera reducción de los valores de selectividad. Sin embargo, la selectividad en la producción con células en *resting* sigue siendo superior a la correspondiente con células en crecimiento, independientemente del medio empleado. La composición del medio no afecta al rendimiento de SA con respecto a la concentración final de biocatalizador ($Y_{SA/X}$) empleando células en crecimiento. Sin embargo, las células en *resting* cultivadas en MPEL parecen presentar vías de metabolismo celular más activas para producir ácido succínico. Similar tendencia se observa para la productividad con células en estado de *resting*, generándose este compuesto 2,4 veces más rápido debido al cambio de la composición del medio (MPEL: 0,43 $g \cdot L^{-1} \cdot h^{-1}$; TSB: 0,18 $g \cdot L^{-1} \cdot h^{-1}$). Además, la productividad específica ($P_{SA/X}$) también se ve favorecida con medio MPEL en la producción con *resting cells*. En definitiva, el medio sintético MPEL apunta a ser una alternativa más prometedora para el desarrollo del bioproceso a escala industrial.

4.4 ESTUDIO DE FORMAS DE OPERACIÓN

En los apartados previos se habían realizado fermentaciones con distintas variables y condiciones de operación, así como estados celulares (en crecimiento o *resting*), pero siempre con el mismo modo de operación: *batch*. En el apartado 1.4.2, se expuso el estado del arte de los distintos tipos de operación estudiados para la bioproducción de ácido succínico, observando que algunos autores habían logrado incrementar significativamente sus rendimientos y/o productividades llevando a cabo fermentaciones en otros modos de operación. Por ello, con el objetivo de mejorar el proceso de producción de ácido succínico, así como de reducir en mayor medida los gastos asociados a las etapas de separación, se decidió llevar a cabo fermentaciones a través de otros dos tipos de operación empleados en bibliografía: *fed-batch* y *repeated batch*, siendo en ambos la primera etapa una operación en *batch* como las ya estudiadas. En este trabajo, cuyos resultados y discusión se presentan en detalle en la **Publicación 4** "*Study on the operational modes using both growing and resting cells for the succinic acid production from xylose. Kinetic modelling*", se compararon los resultados obtenidos con estas dos operaciones empleando células en crecimiento y en estado de *resting.*

4.4.1 *Repeated batch*

Se comenzó estudiando la operación tipo *repeated batch* tanto para células en estado de crecimiento como de *resting*, siendo un tipo de operación que, en principio, permitiría evitar inhibiciones debidas a la acumulación de compuestos citotóxicos en el caldo. Para ello, entre ciclos, se centrifugaron las células procedentes del *batch* anterior y se inocularon al reactor de la etapa siguiente, sumando un total de tres etapas de proceso. Al inicio de cada etapa la cantidad inicial de xilosa fue de 20 g L^{-1}, procurando que la concentración de este azúcar no descendiera más de los 5 g L^{-1}, con el objetivo de evitar un estrés metabólico excesivo. En la Figura 4.7 se muestra la evolución de las concentraciones de xilosa, biomasa, ácido succínico y subproductos en la operación tipo *repeated batch* tanto con células en crecimiento, como en *resting*. En la Tabla 4.7 se presentan los valores de los parámetros de fermentación en cada una de las etapas y en total, de estos procesos.

Tabla 4.7. Concentraciones, selectividad, rendimientos y productividad en la producción de ácido succínico con células en crecimiento y en *resting* operando en *repeated batch*.

Exp.	**33**				**34**			
Biocatalizador	**Células en crecimiento**				***Resting cells***			
Ciclo	**1**	**2**	**3**	**Media**	**1**	**2**	**3**	**Media**
C_{SA} (g_{SA} L^{-1})	8,39	8,56	10,8	9,25	8,13	6,90	1,55	5,53
S_{SA} (g_{SA} g_{SA+SP}^{-1})	0,53	0,52	0,54	0,53	0,65	0,92	0,60	0,72
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,38	0,42	0,54	0,45	0,41	0,35	0,08	0,28
$Y_{SA/S.cons}$ (g_{SA} $g_{S.cons}^{-1}$)	0,50	0,48	0,54	0,51	0,55	0,51	0,30	0,45
$Y_{SA/X}$ (g_{SA} g_X^{-1})	3,21	1,95	1,83	2,33	1,94	2,25	0,71	1,63
P_{SA} (g_{SA} L^{-1} h^{-1})	0,35	0,48	0,77	0,53	0,83	0,18	0,03	0,35
$P_{SA/X}$ (g_{SA} g_X^{-1} h^{-1})	0,13	0,11	0,13	0,12	0,20	0,06	0,01	0,09

En la Figura 4.7 se puede observar que, mientras que, los tres ciclos del *repeated batch,* empleando células en crecimiento, transcurrieron, en total menos de 60 h, cuando se emplearon células en *resting,* el tiempo de fermentación se prolongó 40 h más. Asimismo, en resultados presentados en la Tabla 4.7, se observa que, en el primer ciclo con *resting cells*, la productividad fue más elevada que con células en crecimiento, debido probablemente a la mayor concentración de biocatalizador. En las siguientes etapas, se observa una desaceleración de la velocidad de consumo de sustrato cuando se reutilizan las células en *resting*. Con *resting cells*, la concentración de biocatalizador disminuye progresivamente, debido al procedimiento de recuperación celular experimental entre ciclos. Se observó que, aunque en el segundo ciclo las células seguían considerablemente activas, en el tercer ciclo, el proceso sufrió una importante desaceleración, probablemente causada por el estrés mecánico del proceso de separación del biocatalizador y la escasez de nutrientes durante los ciclos de fermentación consecutivos.

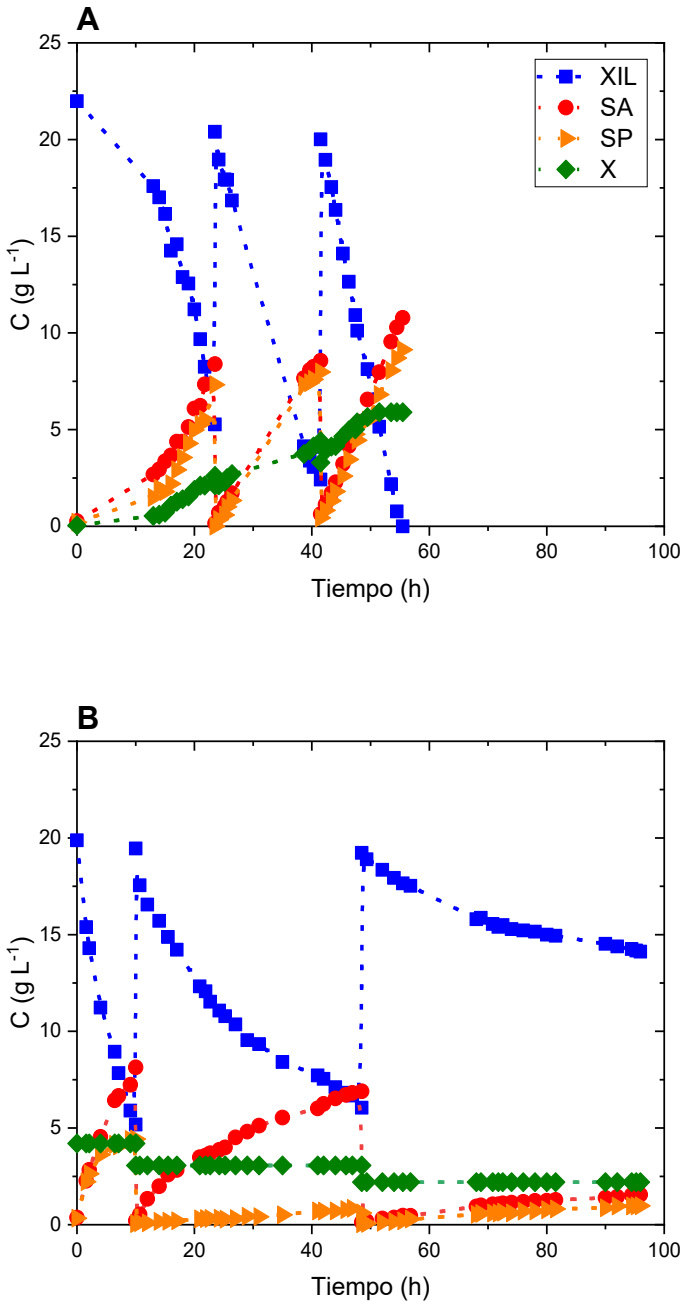

Figura 4.7. Evolución de las concentraciones de sustrato, producto, subproducto y biomasa en una operación tipo *repeated batch* con células en crecimiento – Exp.33 (A) y células en estado de *resting*– Exp.34 (B).

En el caso de trabajar con células en crecimiento, en la Tabla 4.7, se observa que la productividad mejoró a lo largo de los ciclos, tanto en términos de consumo de sustrato como de producción de ácido. También se observa que el rendimiento respecto a la xilosa consumida y la productividad en la tercera etapa son un 72 % y un 56 % superiores a los correspondientes a la primera etapa, respectivamente. Sumando las cantidades finales de

cada una de las etapas de la operación tipo *repeated batch* se logró producir 27,7 g de SA, alcanzando una productividad media de 0,53 g L^{-1} h^{-1}. En cuanto a la productividad en relación a la concentración de biomasa, se observa que las células en estado de crecimiento son más productivas por ciclo que las células en *resting*. Sin embargo, cuando se analiza el valor medio, hay que destacar que se acortan mucho las diferencias en la productividad específica entre ambos estados. En conclusión, aunque la selectividad resultó favorecida por la producción con *resting cells*, la reutilización de células en crecimiento condujo a una mejor producción general de SA, en términos de concentración, rendimiento y productividad.

4.4.2 *Fed-batch*

Posteriormente, se estudió la operación tipo *fed-batch*, tanto con células en crecimiento como en estado de *resting*. Para ello, se llevó a cabo un proceso en tres etapas, partiendo de 20 g L^{-1} de xilosa y llevando a cabo dos alimentaciones de este azúcar concentrado cuando su concentración caía a los 5 g L^{-1}. En la Figura 4.8 se muestra la evolución de las concentraciones de xilosa, biomasa, ácido succínico y subproductos en la operación tipo *fed-batch* tanto con células en crecimiento, como en *resting*. En la Tabla 4.8 se presentan los valores los parámetros de fermentación en cada una de las etapas y en total, de estos procesos.

La duración de las dos primeras etapas de la operación *fed-batch* con células en *resting* fue considerablemente inferior a las correspondientes con células en crecimiento. En ambos casos no se alcanzó el consumo total de sustrato en el último paso. De hecho, en la operación con *resting cells*, después de 30 h de fermentación, sólo se consumió aproximadamente la mitad de la xilosa alimentada en la tercera etapa.

La desaceleración de los procesos en la última parte de los experimentos puede ser causada por muchos factores: el agotamiento de los nutrientes esenciales que no se añaden en la alimentación en pulsos, el envejecimiento de las células, la acumulación de ácidos (tanto el producto objetivo como subproductos) o toxinas con efecto inhibitorio [174]. Esta reducción de la productividad a tiempos largos de fermentación tipo *fed-batch* también ha sido observada por otros autores, como Jiang et al. [173], en cuya fermentación a partir de glucosa advirtieron una caída de la velocidad de crecimiento de *A. succinogenes* y del ácido succínico generado a partir del tercer ciclo de producción (14

h de fermentación), que se volvió especialmente acusada en el cuarto ciclo (iniciado a las 20 h).

Cabe destacar también que, al emplear células en *resting*, la selectividad de SA se potencia por completo, alcanzando una concentración final de SA 3,6 veces superior a la concentración global de subproductos. Sin embargo, en el caso de emplear células en crecimiento, tan solo se alcanza una concentración final de SA 1,4 veces superior a la concentración total de subproductos. Por otro lado, los rendimientos de SA tuvieron una tendencia decreciente entre ciclos en el experimento de células en crecimiento (Ciclo 1: 0,40 g g^{-1}, Ciclo 2: 0,21 g g^{-1}, Ciclo 3: 0,20 g g^{-1}), pero se observó el comportamiento contrario con células en *resting* (Ciclo 1: 0,40 g g^{-1}, Ciclo 2: 0,21 g g^{-1}, Ciclo 3: 0,20 g g^{-1}). La concentración del producto final fue 1,5 veces superior empleando células en *resting* (0,53 g g^{-1}). En ambos casos la productividad se redujo drásticamente a lo largo de los ciclos, con un valor medio superior en el caso de operar con *resting cells* (0,36 g L^{-1} h^{-1}). Estos resultados mostraron que el metabolismo del biocatalizador se ralentizó a lo largo de los ciclos, pero fue más selectivo. La operación en *fed-batch* con células en *resting* conduce a un mayor rendimiento, productividad y selectividad que con células en crecimiento.

Tabla 4.8. Valores de parámetros de fermentación en la producción de ácido succínico con células en crecimiento y en *resting* operando en *fed-batch*.

Exp.	**35**				**36**			
Biocatalizador	**Células en crecimiento**				***Resting cells***			
Ciclo	**1**	**2**	**3**	**Total**	**1**	**2**	**3**	**Total**
C_{SA} (g_{SA} L^{-1})	8,02	3,85	3,64	15,9	7,50	6,99	8,45	22,9
S_{SA} (g_{SA} g_{SA+SP}^{-1})	0,54	0,52	0,76	0,57	0,63	0,87	0,89	0,78
$Y_{SA/Xil.0}$ (g_{SA} $g_{Xil.0}^{-1}$)	0,40	0,21	0,20	0,35	0,40	0,40	0,47	0,53
$Y_{SA/Xil.cons}$ (g_{SA} $g_{Xil.cons}^{-1}$)	0,56	0,30	0,38	0,43	0,56	0,57	1,00	0,68
$Y_{SA/X}$ (g_{SA} g_{X}^{-1})	3,15	1,09	1,03	4,48	1,79	1,74	2,15	5,46
P_{SA} (g_{SA} L^{-1} h^{-1})	0,36	0,23	0,10	0,21	0,83	0,54	0,21	0,36
$P_{SA/X}$ (g_{SA} g_{X}^{-1} h^{-1})	0,14	0,07	0,03	0,06	0,20	0,13	0,05	0,09

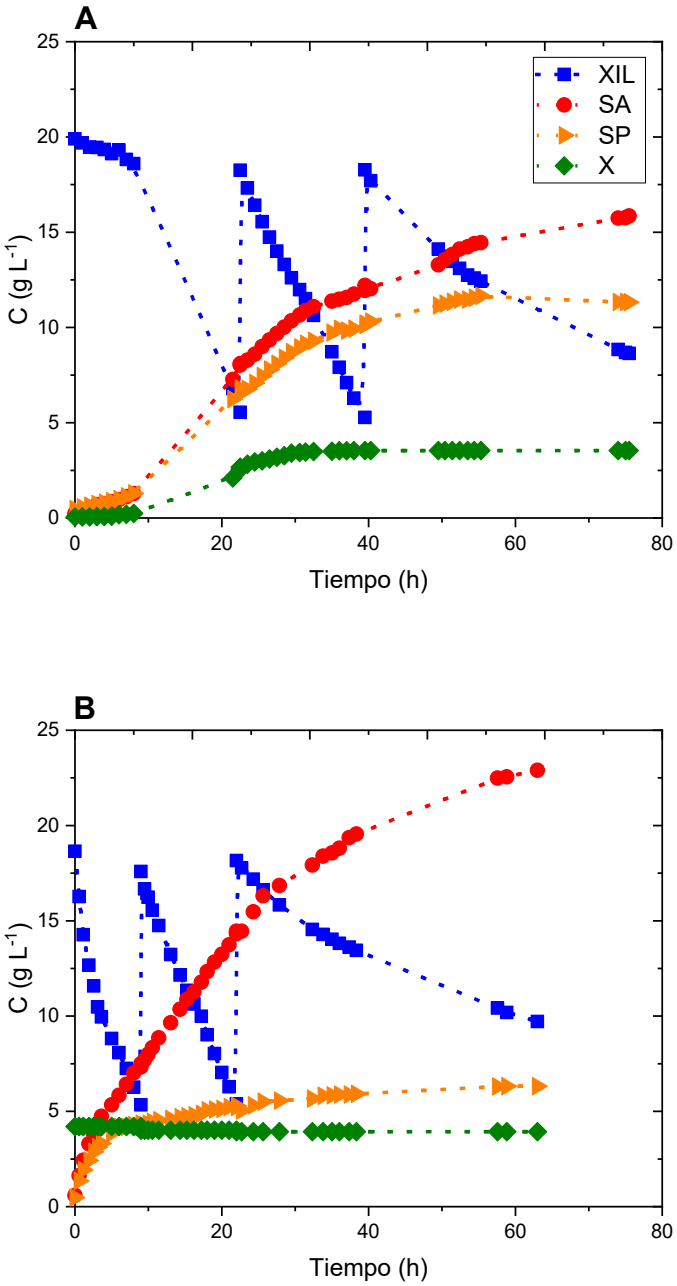

Figura 4.8. Evolución de las concentraciones de sustrato, producto, subproducto y biomasa en una operación tipo *fed-batch* con células en crecimiento – Exp.35 (A) y células en *resting* – Exp.36 (B).

4.5 USO DE HIDROLIZADOS COMO FUENTE DE CARBONO Y DE NITRÓGENO

Para el desarrollo de una bioeconomía circular resultan imprescindibles la reutilización y aprovechamiento de los residuos generados y de materias primas renovables, así como la reducción de los costes asociados a las operaciones de biorrefinería. Una manera de cumplir con ambos requisitos es el aprovechamiento de hidrolizados de residuos como fuente de carbono y/o nitrógeno en las operaciones de fermentación. Por ello, en este apartado se estudiará, por un lado, la utilización de hidrolizados de residuos de patata como sustituto de la glucosa comercial y por otro, el empleo de residuos de una cervecera, tanto hidrolizados de bagazo de cerveza como de levadura de cerveza gastada, como reemplazo de xilosa y extracto de levadura comerciales, respectivamente.

4.5.1 Hidrolizado de levadura de cerveza como fuente de nitrógeno. Optimización de su factor de dilución

En el siglo XXI se ha ido produciendo un gran aumento del consumo de cerveza a nivel mundial debido al impacto en su demanda de las regiones en desarrollo y, por tanto, intensificando la cantidad. de residuos generados por esta industria en crecimiento [175,176], entre los cuales cabe destacar la levadura de cerveza gastada, un residuo de elevado valor proteico. Anualmente se generan en torno a 0,125 millones de toneladas de levadura de cerveza gastada [177], por lo que se decidió estudiar el empleo de un hidrolizado de este desecho, procedente de la empresa cervecera La Cibeles S.L (Leganés, Madrid) como fuente de nitrógeno en la producción de ácido succínico con *A. succinogenes*. Evitar el consumo de fuentes de nutrientes comerciales, como el extracto de levadura (EL), permitiría el ahorro de uno de los principales costes asociados al proceso de fermentación. Los resultados de los experimentos realizados a partir de residuos de cervecera se recogen en la publicación **Publicación 6** "*Integral use of brewery wastes as carbon and nitrogen sources for the bioproduction of succinic acid*".

Jiang et al. [133] llevaron a cabo un estudio en el que utilizaron un hidrolizado de levadura de cerveza gastada (HLCG) suplementado con vitaminas como fuente de nitrógeno. Utilizaron la cantidad suficiente como para que la concentración de nitrógeno

total fuera la misma que cuando empleaban EL. En los experimentos llevados a cabo por estos autores los rendimientos con el hidrolizado fueron inferiores a cuando se utilizó la fuente de nitrógeno comercial. Por ello, en el presente trabajo, considerando más importante la biodisponibilidad de la fuente de nitrógeno que la propia cantidad en sí, se realizaron 5 experimentos en botella con distintos factores de dilución (FD) de HLCG (sin suplementación de vitaminas) y se compararon con un sexto experimento en botella con 10 g L^{-1} de EL, empleando 20 g L^{-1} de xilosa como fuente de carbono en todos los casos. Los resultados de los valores de los parámetros de estas producciones se muestran en la Tabla 4.9, en la que se puede observar que el incremento del factor de dilución condujo a un incremento de las selectividades A pesar de esto, los valores más altos de rendimiento y productividad no se alcanzaron con el factor de dilución más bajo (FD 12), sino con el siguiente que se estudió (FD 6). Al utilizar un FD 12, el rendimiento de ácido succínico en relación a la xilosa consumida fue muy similar al correspondiente a la fermentación con FD 6. Sin embargo, las diferencias entre los rendimientos de ácido succínico en función de la cantidad de xilosa inicial son mucho mayores (16 % para FD 12 y 23 % para FD 6), lo que parece indicar una cantidad insuficiente de nutrientes con un FD 12. Los valores de rendimiento y productividad con FD 4,5, FD 3 y FD 2 fueron incluso inferiores a los obtenidos con FD 6. Teniendo en esto en cuenta, que con FD 6 la cantidad de subproductos es inferior y además se precisa una menor cantidad de hidrolizado que a FD mayores, se concluye que este último FD es el óptimo para producir ácido succínico.

Tabla 4.9. Valores de parámetros de fermentación después de 24 h de fermentación en botella con HLCG a diferentes FD y EL comercial como fuentes de nitrógeno.

Exp.	**37**	**38**	**39**	**40**	**41**	**42**
Fuente nitrógeno	**HLCG**					**EL (10 g L^{-1})**
Factor de dilución	12	6	4,5	3	2	1
C_{SA} (g L^{-1})	3,45	4,41	4,17	3,98	3,69	4,39
S_{SA} (g g^{-1})	0,69	0,67	0,61	0,40	0,36	0,52
Y_{SA} (g g^{-1})	0,16	0,23	0,21	0,21	0,18	0,22
$Y_{SA/S.cons}$ (g g^{-1})	0,58	0,60	0,54	0,38	0,33	0,60
P_{SA} (24 h) (g L^{-1} h^{-1})	0,14	0,18	0,17	0,17	0,15	0,18

4.5.2 Producción en operación tipo *batch* con hidrolizado de levadura de cerveza gastada como fuente de nitrógeno

Una vez determinada la cantidad de HLCG adecuada, se llevaron a cabo experimentos en reactores de 2 L, a partir de 20 g L^{-1} de xilosa con este hidrolizado. En la Figura 4.9 se muestra la evolución del sustrato (xilosa), biomasa, ácido succínico y subproductos (ácidos acético y fórmico) a lo largo del tiempo de esta fermentación en comparación con su equivalente realizada con EL comercial.

El consumo de xilosa al utilizar EL es más rápido (Figura 4.9 B), de hecho, su fermentación finaliza prácticamente 70 h antes que con HLGC. Cabe señalar que el crecimiento del microorganismo (Figura 4.9 C) no se ve afectado por la fuente de nitrógeno utilizada, realizándose a la misma velocidad en los dos experimentos realizados. Con respecto a la producción de ácidos (Figura 4.9 A), la utilización del hidrolizado como fuente de nitrógeno redujo significativamente la producción de subproductos (alcanzando una selectividad del 68 %, lo que implica un aumento del 36 % respecto a la del proceso equivalente con EL. Por tanto, aunque ralentice el proceso, la concentración de ácido succínico y rendimientos alcanzados son superiores (12,8 g L^{-1}; 0,61 g g^{-1}) que en la fermentación con EL (10,6 g L^{-1}; 0.53 g g^{-1}). La productividad con HLGC (0,14 g L^{-1} h^{-1}) resultó ser algo menos de la mitad que con EL (0,38 g L^{-1} h^{-1}).

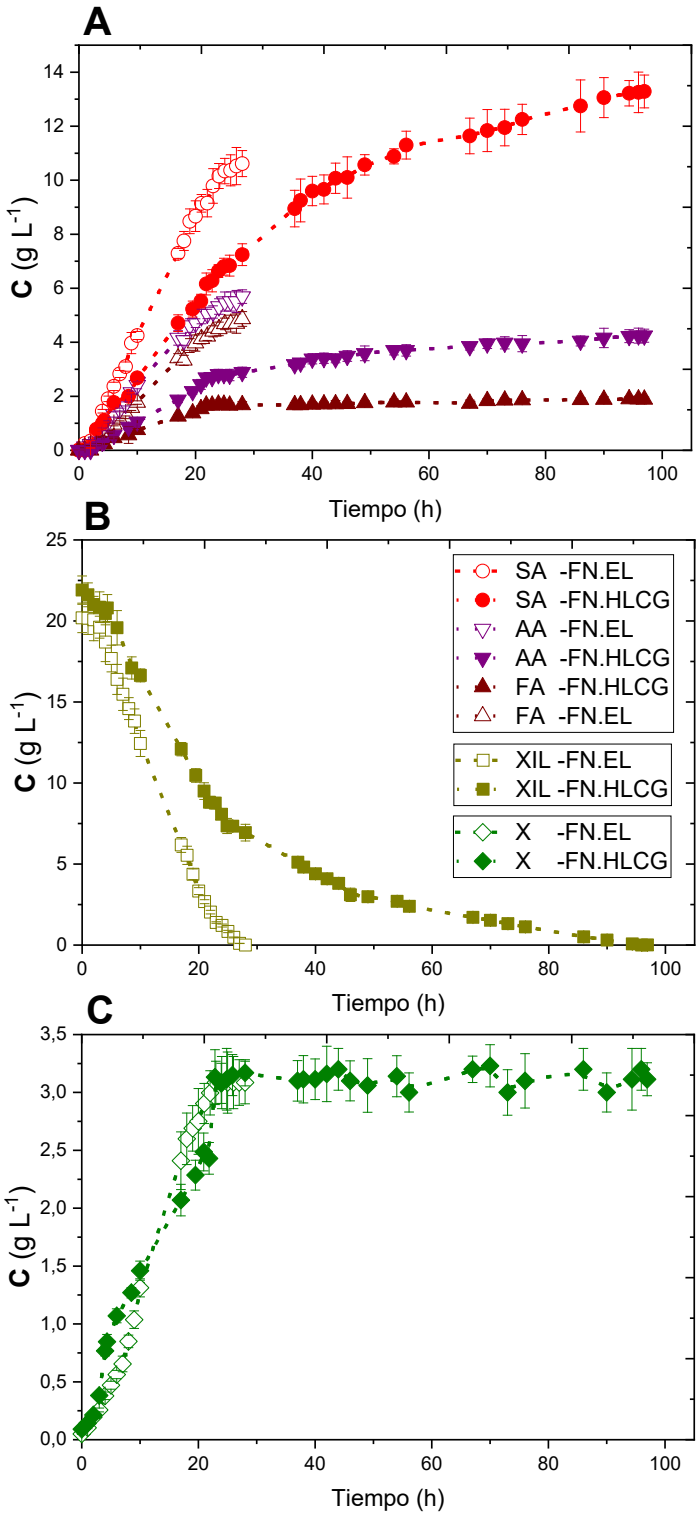

Figura 4.9. Evolución temporal de ácidos succínico, acético y fórmico (gráfico A), xilosa (gráfico B) y biomasa (gráfico C) en fermentaciones en *batch* cuya fuente de nitrógeno (FN) fue EL comercial (Exp.43) o HLCG (Exp.44).

4.5.3 Producción en operación tipo *batch* con hidrolizado de bagazo de cerveza como fuente de carbono

El bagazo de cerveza representa el 85 % del total de residuos sólidos en la industria cervecera, con una producción anual total de 34-35 millones de toneladas en Europa [177]. En este trabajo, tras la hidrólisis enzimática de este residuo (apartado 3.1.1) en el que, tras la acción de la α-amilasa, gracias a la cual se hidrolizaron los enlaces α.1,4 glicosídicos de la amilosa y la amilopectina liberando dextrinas y oligosacáridos, fue posible aplicar un cóctel de endo-βglucanasas que hidrolizaron enlaces (1,3) o (1,4) en β-D-glucanos y xilanasas que hidrolizaron enlaces (1,4)-β-D-xilosídicos en xilanos. La endoproteasas hidrolizaron enlaces péptidos internos y las glucoamilasas rompieron las dextrinas en azúcares sencillos. Finalmente, gracias a una mezcla de endo- y exo-glucanasas, se logró la escisión del polímero de celulosa en azúcares más pequeños y polisacáridos oligoméricos, obteniendo un hidrolizado (HBC) cuya composición se especifica en la Tabla 4.10. El hidrolizado de bagazo de cerveza (HBC) se empleó como fuente de carbono, lo suficientemente diluido como para preparar un medio de cultivo con 20 g L^{-1} de xilosa, en la producción de ácido succínico en reactor de 2 L. Se utilizó EL comercial como fuente de nitrógeno. Los resultados de estos experimentos con HBC se compararon con los obtenidos en la operación equivalente con 20 g L^{-1} de xilosa pura. La evolución de los sustratos, ácido succínico, subproductos y biomasa en ambos en casos se refleja en la Figura 4.10.

Tabla 4.10. Concentraciones de azúcares en el hidrolizado de bagazo de cerveza (HBC).

Compuesto	**Concentración (g L^{-1})**
Sacarosa (SAC)	0,00
Maltosa (MALT)	1,57
Glucosa (GLUC)	0,57
Xilosa (XIL)	105
Galactosa (GALACT)	11,8
Arabinosa (ARAB)	0,19
Manosa (MAN)	3,87
Fructosa (FRUCT)	1,56

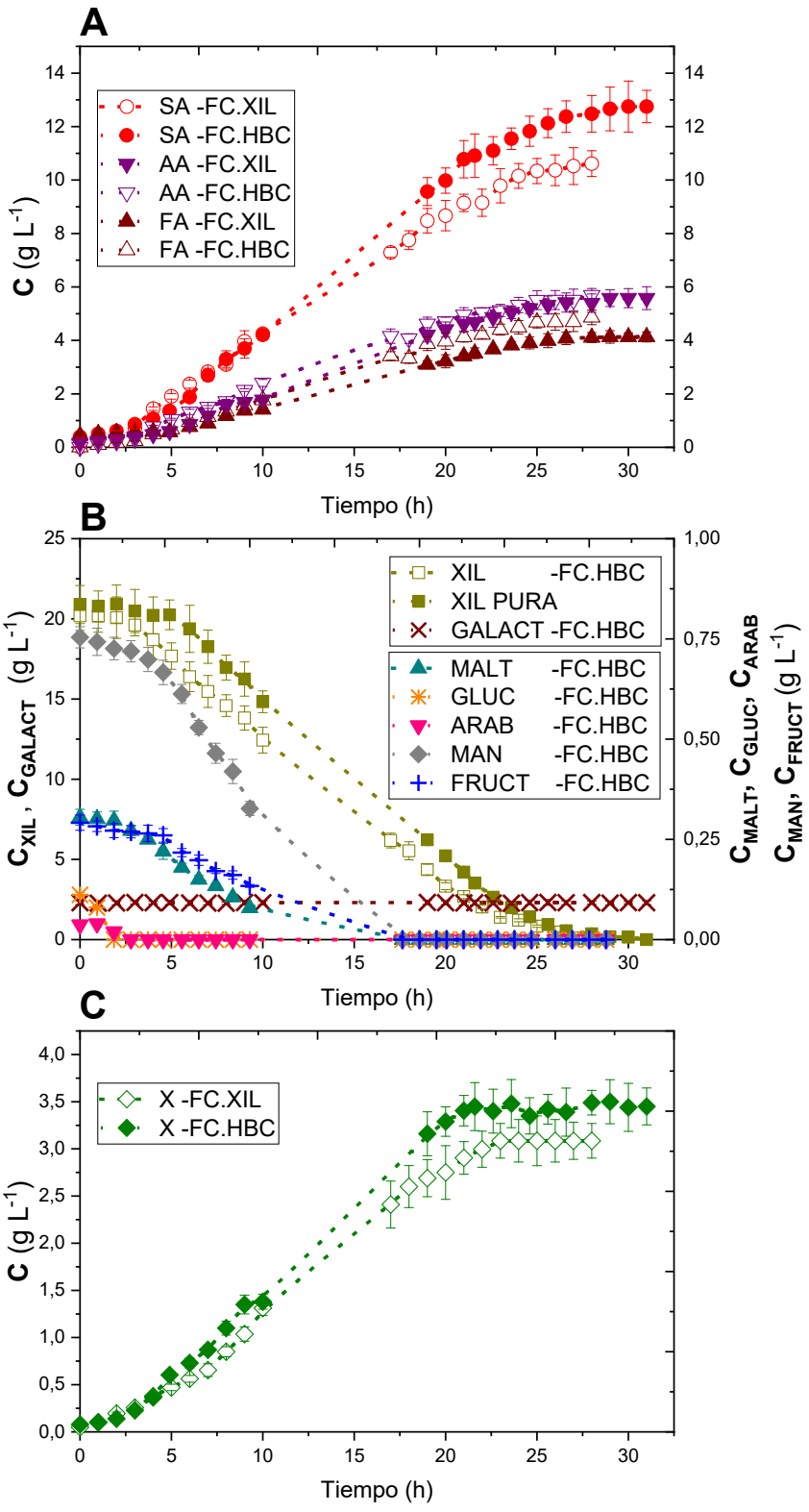

Figura 4.10. Evolución temporal de ácidos succínico, acético y fórmico (A), xilosa (B) y biomasa (C) en fermentaciones en *batch* cuya fuente de carbono (FC) fue xilosa pura (Exp.43) o HBC (Exp.45).

En la citada figura se puede observar que la producción con HBC, aquellos azúcares que no son xilosa (maltosa, glucosa, arabinosa, manosa y fructosa) y que, por tanto, se encuentran en baja cantidad, se consumen por completo alrededor de 17 h después del inicio de la fermentación. Sin embargo, el microorganismo no es capaz de metabolizar la galactosa. En cuanto al consumo de xilosa (Figura 4.10.B), como reactivo puro es algo más rápido, pero sin grandes diferencias con la xilosa procedente del hidrolizado, agotándose en ambos casos alrededor de las 27 h. La producción de ácido succínico que se logra alcanzar (ver Figura 4.10.A) es mayor cuando se utiliza HBC (13,3 g L^{-1}), aunque no es muy superior a la que se obtiene con 20 g L^{-1}de xilosa pura (10,6 g L^{-1}), tal y como se muestra, más adelante, en la Tabla 4.11. En cuanto a los dos subproductos obtenidos en el proceso (ácido fórmico y ácido acético), no se observa diferencia significativa entre el uso de xilosa pura o HBC. En cuanto a la productividad, al utilizar HBC su valor sufre un ligero incremento respecto al obtenido con xilosa pura (0,41 versus 0,38 g L^{-1} h^{-1}). También se determina que el uso de HBC favorece la selectividad de producción de ácido succínico, siendo del 57 % con este residuo frente al 50 % en el caso de utilizar xilosa comercial.

4.5.4 Producción en operación tipo *batch* con hidrolizado de bagazo de cerveza como fuente de carbono e hidrolizado de levadura de cerveza gastada como fuente de nitrógeno

Con el objetivo de evitar completamente el empleo de fuentes de carbono o nitrógeno comerciales, se llevaron a cabo experimentos de fermentación con HBC en sustitución de la xilosa pura y HLGC para reemplazar el EL. En la Figura 4.11 se recoge la evolución a lo largo del tiempo de la concentración del sustrato, ácido succínico, subproductos y biomasa en estas fermentaciones. En la Tabla 4.11 se muestra una tabla comparativa de los resultados obtenidos en los experimentos realizados con HBC o xilosa pura y HLGC o EL comercial.

En primer lugar, en la Figura 4.11, se puede comprobar que la realización del proceso de fermentación con ambos residuos de manera simultánea fue viable, obteniendo una concentración de ácido succínico de 15,6 g L^{-1}, valor superior a los obtenidas en el resto de los experimentos realizados (Tabla 4.11). Asimismo, como se observó en la producción llevada a cabo con HBC y LE (Figura 4.10, apartado 4.5.3), la productividad

se redujo aproximadamente a la mitad de la observada cuando se utilizó xilosa pura; sin embargo, el rendimiento de ácido succínico obtenido (0,77 g g^{-1}) fue el más alto de todos los experimentos realizados, incluido el experimento empleado como referencia sin residuos (0,53 g g^{-1}), como se muestra en la Tabla 4.10. Teniendo en cuenta que, como se mencionó anteriormente, los elevados costes de la purificación de ácido succínico son una de los principales obstáculos en su bioproducción [169], vale la pena señalar que el uso simultáneo de los residuos de la cervecería como fuente de carbono y nitrógeno, en lugar de xilosa comercial y EL, implica duplicar la selectividad del proceso, alcanzando un valor del 76 %.

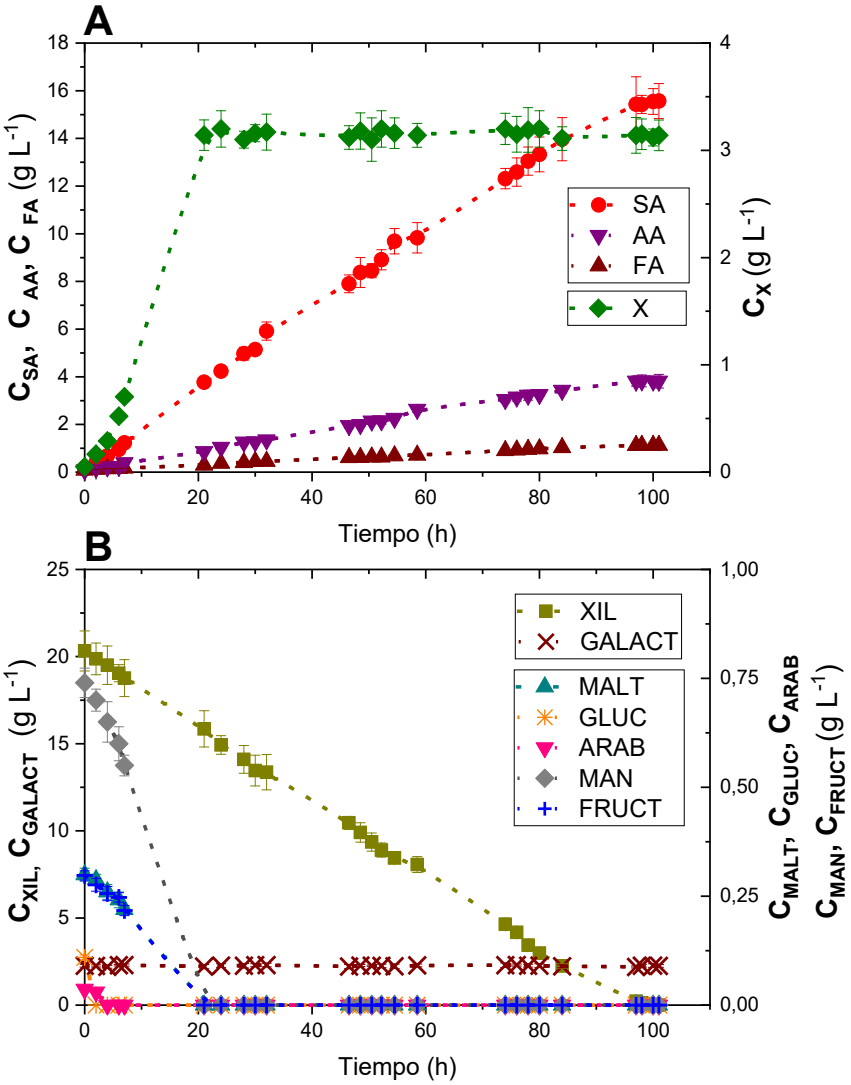

Figura 4.11. Evolución temporal de ácidos succínico, acético y fórmico (A), xilosa (B) y biomasa (C) en fermentaciones con HBC como fuente de carbono y HLGC como fuente de nitrógeno en operaciones tipo *batch* –Exp.46.

Tabla 4.11. Valores de parámetros de fermentación a partir de xilosa pura o HBC como fuente de carbono y EL o HLGC como fuente de nitrógeno en operaciones tipo *batch.*

Exp.	**43**	**44**	**45**	**46**
Fuente carbono	**Xilosa pura**		**HBC**	
Fuente nitrógeno	**EL**	**HLGC**	**EL**	**HLGC**
C_{SA} (g L^{-1})	10,6	12,8	13,3	15,6
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,53	0,61	0,61	0,77
$Y_{SA/S.cons}$ (g_{SA} $g_{S.cons}^{-1}$)	0,53	0,61	0,61	0,77
P_{SA} (g_{SA} L^{-1} h^{-1})	0,38	0,14	0,41	0,15
S_{SA} (g_{SA} g_{SA+SP}^{-1})	0,50	0,68	0,57	0,76

4.5.5 Residuos de patata como fuente de carbono. Determinación del tiempo óptimo de hidrólisis ácida.

Según la FAO, en 2019, la patata fue el cuarto producto agrícola más importante a nivel mundial [178]. Por lo que, teniendo en cuenta que se desperdicia en torno al 15-40% de su peso, anualmente se desperdician entre 55 y 140 millones de toneladas [179]. En este trabajo se llevó a cabo el aprovechamiento de trozos de patata desechados en una fábrica de *snacks*, ESPAFRIMA S.L (Getafe, Madrid), como fuente de glucosa en la producción de ácido succínico. Los resultados de estos experimentos, junto a su discusión comparativa con trabajos realizados por otros autores sobre fermentación de ácido succínico a partir de hidrolizados se recogen en la **Publicación 5** "*Bioproduction of succinic acid from potato waste: process development and kinetic modeling*".

Los residuos de patata fueron tratados mediante hidrólisis ácida, según se recoge en el apartado 3.3.1 del capítulo de Materiales y Métodos de la presente Memoria. La evolución de la glucosa (sustrato, S) y 5-hidroximetilfurfural (5-HMF) a lo largo de este proceso se muestra en la Figura 4.12.

En la Figura 4.12 se puede observar que, durante los primeros 40 minutos de hidrólisis se liberó glucosa, a partir de amilosa y amilopectina, a una velocidad de 1,8 g L^{-1} min^{-1}. A partir de ese momento, esta productividad disminuyó progresivamente. La velocidad de deshidratación de la glucosa, generando 5-HMF, se incrementó a partir de los 20 minutos de hidrólisis. A los 60 minutos de proceso, la diferencia entre la concentración de glucosa y 5-HMF fue máxima, manteniéndose una concentración de 5-HMF relativamente baja. Por tanto, para evitar la acción deletérea de este subproducto

sobre *A. succinogenes* durante la fermentación [180], se eligió un tiempo de hidrólisis de 60 minutos, alcanzando una concentración de glucosa de 66,5 g L^{-1} y 0,11 g L^{-1} de HMF.

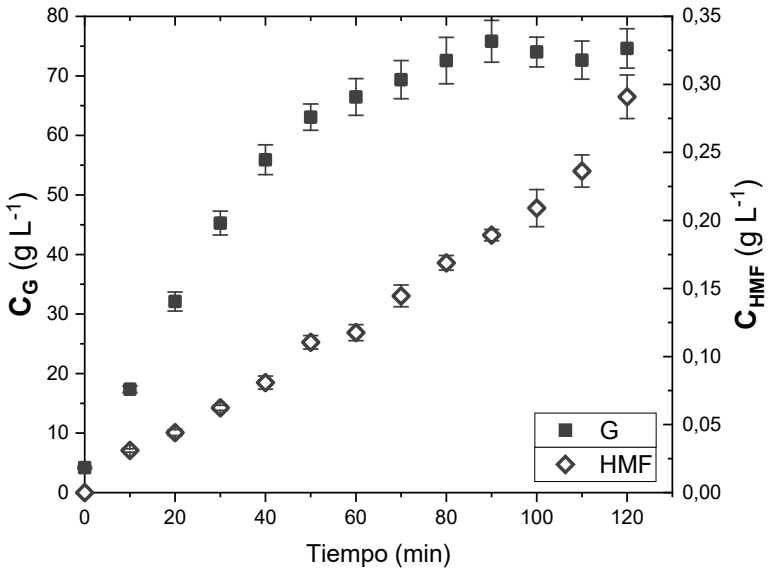

Figura 4.12. Evolución en el tiempo de la concentración de glucosa (G) y de HMF durante la hidrólisis ácida de residuos de patata.

4.5.6 Producción de ácido succínico a partir de hidrolizados de residuos de patata en botella

Tras obtener un hidrolizado, por tratamiento ácido, rico en glucosa, se empleó la cantidad suficiente de este como para llevar a cabo experimentos en los que se partiera de una concentración inicial de glucosa de 40 g L^{-1}. Se comenzó estudiando la producción de ácido succínico con *A. succinogenes* con esta fuente de carbono (hidrolizado de residuos de patata, HRP) en fermentaciones en botella, comparando los resultados con el empleo de glucosa pura como fuente de carbono, como se muestra en la Tabla 4.12.

Tabla 4.12. Valores de parámetros de fermentación en la producción a partir de glucosa pura y HRP en botellas.

Exp.	**47**	**48**
Sustrato	**Glucosa pura**	**HRP**
C_{SA} (g L^{-1})	11,3	10,6
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,28	0,27
$Y_{SA/S.cons}$ (g_{SA} $g_{S.cons}^{-1}$)	0,53	0,50
P_{SA} (g_{SA} L^{-1} h^{-1})	0,46	0,32
S_{SA} (g_{SA} g_{SA+SP}^{-1})	0,55	0,46

Observando los datos de la citada tabla, se puede decir que, a pesar de que en botella no se logren elevados rendimientos, independientemente de la fuente de carbono utilizada, estos experimentos sirvieron como prueba de concepto a la hora de comprobar la viabilidad del empleo de este hidrolizado, lo cual se confirmó al apreciar que los rendimientos y concentraciones finales de ácido succínico fueron muy similares con los dos sustratos empleados. Ha de mencionarse que, en el caso de utilizar el HRP, la productividad disminuyó en un 38 % y la selectividad en un 18 %.

4.5.7 Producción de ácido succínico a partir de residuos de patata en operación tipo *batch*

Posteriormente, se llevaron a cabo experimentos en reactor de 2 L llevando a cabo una operación tipo *batch* con HRP, en una cantidad suficiente como para iniciar la fermentación con una concentración inicial de glucosa de 40 g L^{-1} aproximadamente. Los valores de parámetros de fermentación de este proceso de producción de ácido succínico a partir de residuos de patata se presentan en la Tabla 4.13 (Exp. 50), donde se comparan con su experimento equivalente llevado a cabo a partir de 40 g L^{-1} de glucosa pura (Exp. 49).

Tabla 4.13. Valores de parámetros de fermentación en la producción en reactor tipo *batch* a partir de 40 g L^{-1} de glucosa pura y HRP.

Exp.	**49**	**50**
C_{SA} (g L^{-1})	27,4	32,2
$Y_{SA/S.0}$ (g_{SA} $g_{S.0}^{-1}$)	0,68	0,92
$Y_{SA/S.cons}$ (g_{SA} $g_{S.cons}^{-1}$)	0,68	0,95
P_{SA} (g_{SA} L^{-1} h^{-1})	0,83	0,64
S_{SA} (g_{SA} g_{SA+SP}^{-1})	0,62	0,80

Como se observa en la Tabla 4.13, al emplear el hidrolizado se consiguió alcanzar una concentración final de ácido succínico de 32,2 g L^{-1}. Este experimento permitió el incremento del rendimiento de SA (0,92 g g^{-1}) en un 37 % con respecto a su experimento equivalente con glucosa pura, así como aumentar la selectividad en un 22,5 % (alcanzando un valor de 0,80 g g^{-1}), lo cual es de gran relevancia teniendo en cuenta los elevados costes de purificación de SA, tal y como se mencionó en el apartado 4.3. Sin embargo, cabe destacar que la velocidad de producción sufrió una disminución del 24 %, probablemente debido a la presencia de cantidades suficientes 5-HMF como para generar

ligeros efectos citotóxicos o la necesidad de realizar un número todavía mayor de etapas de adaptación previas al hidrolizado.

4.5.8 Producción en *repeated batch* con a partir de residuos. Hidrolizado de patata como fuente de carbono e hidrolizado de levadura de cerveza gastada como fuente de nitrógeno

Hasta el momento, sólo se había operado en modo *batch* al emplear hidrolizados de residuos como fuente de carbono o nitrógeno. En el apartado 4.3, al comparar las operaciones *fed-batch* y *repeated batch* para la producción de succínico a partir de xilosa con *resting cells* y con células en crecimiento, se determinó que, cuando se trabajaba con células en crecimiento, la operación que permitía alcanzar mayores rendimientos era *repeated batch*.

En el apartado 4.5.7 se habían llevado a cabo fermentaciones tipo *batch* empleando un HRP, rico en glucosa, como fuente de carbono, obteniendo rendimientos y selectividades un 37 % y un 22 % superiores a los obtenidos en la operación equivalente con glucosa pura, se decidió optimizar la producción a partir de hidrolizado de residuos de patata (HRP) con una operación tipo *repeated batch* con extracto de levadura (EL) como fuente de nitrógeno. En el apartado 4.5.2, se pudo comprobar que el empleo de hidrolizado de levadura de cerveza gastada (HLCG) como sustituto del extracto de levadura (EL) en una operación tipo *batch* con xilosa no solo era viable, sino que permitía obtener ácido succínico con un rendimiento y selectividad un 21 % y 36 % superiores a los de su proceso equivalente con EL comercial, aunque precisaba una mayor prolongación en los tiempos de fermentación. Con el objetivo de, posteriormente, poder llevar a cabo una fermentación tipo *repeated batch* con sustitución simultánea de las fuentes de carbono y nitrógeno por estos residuos, primero, se decidió comprobar la viabilidad de producir ácido succínico en una operación tipo *repeated batch* con cada uno de estos hidrolizados de manera aislada. Los resultados de estos experimentos, junto con aquellos en los que se logró la sustitución integral de las fuentes de carbono y nitrógeno operando en *repeated batch* se muestran en la Figura 4.13 y la Tabla 4.14, donde además se comparan sus rendimientos y productividades con la operación equivalente con glucosa pura y extracto de levadura (EL) comercial.

La discusión de estos experimentos realizados a partir de residuos con operaciones tipo *repeated batch*, se recogen en la **Publicación 7** "*Succinic acid production by Actinobacillus succinogenes using acid and enzymatic hydrolysates of potato and beer wastes and repeated batch operation*",

En la Tabla 4.14, se puede observar que tanto las producciones obtenidas en operación tipo *repeated batch* en las que sólo se sustituyó la fuente de carbono por HRP, el rendimiento, la concentración final de succínico y la productividad aumentaron en cada etapa, alcanzando un máximo de 89 %, 31,1 g L^{-1} y 2,83 g L^{-1} h^{-1}, respectivamente. A pesar de ello, la selectividad disminuyó ligeramente en cada etapa del proceso, pasando del 78 % en la primera etapa al 74 % en la tercera. En el apartado 4.5.1, se había observado que la adición de etapas de adaptación a la fuente de carbono en botella favorecía la resistencia del microorganismo a la centrifugación y permitía operar en estado de *resting*, por ello, podría deducirse que la realización de varias etapas del proceso podría asemejarse a estas adaptaciones previas a la fermentación en reactor y, por tanto, ser favorable para la producción de ácido succínico. También se observa que los rendimientos y productividades con relación a la concentración de biomasa alcanzan su máximo en la segunda etapa.

En la citada tabla se observa también que, al sustituir la fuente de nitrógeno comercial por HLCG, el rendimiento máximo se alcanzó en la tercera etapa (81 %), pero la productividad máxima se alcanzó en la segunda (1,93 g L^{-1} h^{-1}). La selectividad en las dos primeras etapas fue muy similar, alrededor de 78 %, mientras que en la tercera etapa disminuyó a 74 %. Debido al aumento de la densidad celular a lo largo de las etapas, en la primera etapa se logró el valor máximo de rendimiento en relación con la concentración de biomasa. Sin embargo, la actividad metabólica parece haber sido máxima en la segunda etapa, por lo que la máxima productividad en relación con la concentración celular se alcanzó en también en este momento de la fermentación. A pesar de utilizar una fuente de nutrientes diferente a la del experimento del apartado anterior (4.5.7) (HLGC en lugar de EL), el comportamiento de la biomasa fue muy similar en ambos casos, alcanzando un máximo en su concentración a las pocas horas del inicio de la fermentación y manteniéndose constante hasta el inicio de la segunda etapa. En las etapas segunda y tercera, la concentración celular no dejó de crecer hasta el final del proceso, probablemente debido a la mayor productividad de estas etapas, evitándose el agotamiento de los nutrientes en el caldo.

Tabla 4.14. Valores de parámetros de fermentación en la producción de ácido succínico en modo *repeated batch* en función de la fuente de carbono (FC) (glucosa o HRP) y la fuente de nitrógeno (FN) (EL o HLGC).

	FC: GLUCOSA				FC: HRP				
Ciclo	**1**	**2**	**3**	**Total**	**1**	**2**	**3**	**Total**	
Exp.		**51**				**52**			**FN: EL**
C_P (g_P L^{-1})	23,8	25,4	24,8	24,7	27,7	28,1	31,1	28,8	
S_P (g_P g_{P+SP}^{-1})	0,62	0,62	0,59	0,61	0,78	0,77	0,74	0,76	
Y_P (g_P $g_{S.0}^{-1}$)	0,60	0,65	0,62	0,62	0,74	0,74	0,81	0,76	
$Y_{P/S.cons}$ (g_P $g_{S.cons}^{-1}$)	0,68	0,77	0,62	0,69	0,87	0,86	0,89	0,87	
$Y_{P/X}$ (g_P g_X^{-1})	4,66	2,54	2,02	3,07	4,13	2,67	2,35	3,05	
P_P (g_P L^{-1} h^{-1})	0,88	2,99	3,10	2,32	0,92	2,34	2,83	2,03	
$P_{P/X}$ (g_P g_X^{-1} h^{-1})	0,17	0,30	0,25	0,24	0,14	0,22	0,21	0,19	
Exp.		**53**				**54**			**FN: HLGC**
C_P (g_P L^{-1})	29,1	32,2	33,1	31,5	30,2	34,7	35,8	33,6	
S_P (g_P g_{P+SP}^{-1})	0,78	0,79	0,74	0,77	0,80	0,81	0,78	0,80	
Y_P (g_P $g_{S.0}^{-1}$)	0,73	0,80	0,81	0,78	0,77	0,84	0,90	0,84	
$Y_{P/S.cons}$ (g_P $g_{S.cons}^{-1}$)	0,87	0,92	0,85	0,88	0,89	0,97	0,94	0,93	
$Y_{P/X}$ (g_P g_X^{-1})	5,81	3,39	2,82	4,01	5,93	3,53	2,94	4,13	
P_P (g_P L^{-1} h^{-1})	0,63	1,93	1,42	1,32	0,59	1,72	1,28	1,19	
$P_{P/X}$ (g_P g_X^{-1} h^{-1})	0,13	0,20	0,12	0,15	0,12	0,17	0,11	0,13	

En la Tabla 4.14 se observa que, al hacer la sustitución simultánea de las fuentes de carbono y de nitrógeno, de nuevo, el rendimiento de la fermentación resultó favorecido por la realización de sucesivas etapas. Tal y como se comentó anteriormente, probablemente la realización de sucesivas etapas mejore la adaptación del microorganismo a la fuente de carbono. El valor máximo de rendimiento se alcanzó en la última de las etapas (90 %). Sin embargo, la velocidad de producción fue mayor en la segunda etapa (1,72 g L^{-1} h^{-1}). El mayor rendimiento en relación a la concentración celular fue el correspondiente a la primera etapa, mientras que la máxima productividad en relación a la biomasa se obtuvo en la segunda etapa.

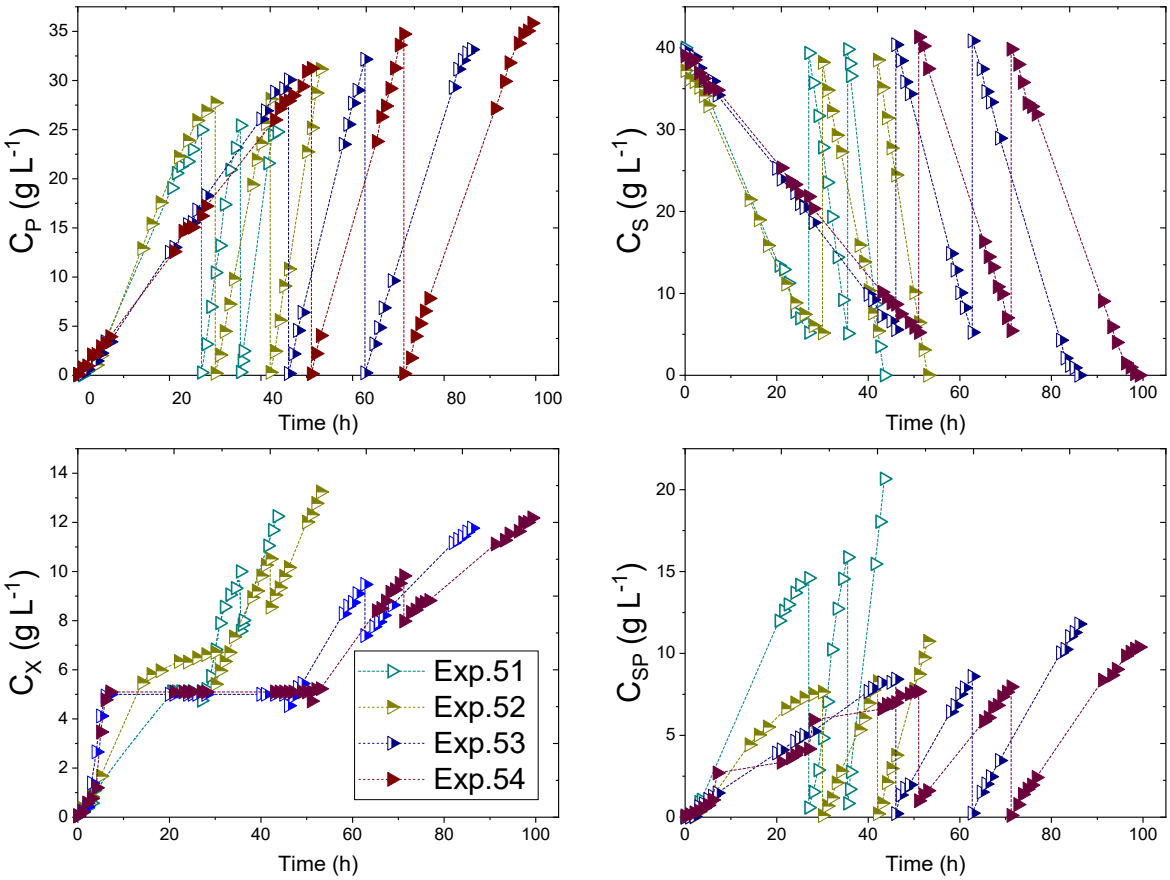

Figura 4.13. Evolución de la concentración de la glucosa (G), ácido succínico, subproductos (ácidos acético y fórmico) y biomasa a lo largo del tiempo en las fermentaciones tipos *repeated batch* a partir de glucosa pura o HRP y EL o HLCG.

Al comparar los resultados generales de los experimentos realizados en *repeated batch,* recogidos en la Tabla 4.14, con las distintas combinaciones de fuentes de carbono y nitrógeno puras o procedentes de residuos, se puede concluir que el uso de hidrolizados, parece favorecer el rendimiento del proceso. De hecho, cuando se utilizó glucosa pura y EL, el rendimiento global del proceso con respecto al sustrato consumido fue de 69 %, mientras que cuando se reemplazaron simultáneamente por residuos, este valor aumentó a un 93 %. Sin embargo, con la productividad la tendencia fue la contraria, en caso de utilizar nutrientes comerciales la productividad promedio fue de 2,32 g L^{-1} h^{-1}, mientras que cuando se utilizaron HRP y HLGC disminuyó a 1,19 g L^{-1} h^{-1}. Esta caída en la productividad fue especialmente pronunciada al reemplazar la fuente de nitrógeno, disminuyendo en un 43 %, cuando al reemplazar exclusivamente la fuente de carbono, la productividad solo se había reducido en un 12,5 %. Cabe señalar que, en el experimento

en el que se sustituyó el azúcar comercial por HRP, el rendimiento y la productividad del proceso se vio claramente favorecido al realizar sucesivas etapas de fermentación, a pesar del estrés que generaba la centrifugación entre etapas, alcanzando el máximo rendimiento y productividad al final de la fermentación. Sin embargo, al utilizar HLGC en vez de EL, a pesar de que el rendimiento fue máximo en la segunda etapa, la productividad del proceso disminuyó en la tercera etapa, revelando una disminución en el ritmo metabólico del microorganismo.

4.6 INFLUENCIA DEL APORTE DE CO_2 EN EL PROCESO DE PRODUCCIÓN DE ÁCIDO SUCCÍNICO

Como se ha comentado en la introducción de esta Memoria, el proceso de producción de ácido succínico por fermentación a partir de azúcares requiere de cierto aporte de CO_2. Este aporte se suele llevar a cabo a nivel de incubadora mediante la introducción de carbonatos que se van transformando en dióxido de carbono en función del pH que se va generando en el medio de cultivo debido al avance del proceso fermentativo. Una vez que el proceso se traslada a biorreactor, la introducción de este compuesto se lleva a cabo de forma directa mediante el burbujeo de dióxido de carbono en forma gaseosa. Aunque en la literatura hay ciertos estudios y modelos que incorporan este sustrato gaseoso en la descripción del proceso, no se ha encontrado ningún trabajo en el que se aborde el estudio de la transferencia y consumo de dióxido de carbono en este proceso, y su posible influencia en la mejora de los parámetros fermentativos del mismo.

En primer lugar, debido a que el dióxido de carbono forma parte de dos equilibrios ácido-base en serie, para considerar la concentración total disuelta del mismo, es necesario tener en cuenta la concentración de todos los compuestos que forman parte de los citados equilibrios. Para poder determinar las citadas concentraciones a lo largo de este estudio, se consideran las siguientes reacciones (Ecuaciones 4.1 – 4.4) y constantes de equilibrio a la temperatura y pH de operación empleados en este trabajo 37 ºC:

$$CO_2(g) \leftrightarrow CO_2(l) \therefore K_H = \frac{[CO_2(l)]}{p_{CO_2}(g)} = 10^{-1,607}\ mol\ L^{-1}\ atm^{-1} \qquad \text{(Ec.4.1)}$$

$$CO_2(l) + H_2O \leftrightarrow H_2CO_3 \therefore K_M = \frac{[H_2CO_3]}{[CO_2(l)]} = 10^{-2,8} \qquad \text{(Ec.4.2)}$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \therefore K_{a1} = \frac{[HCO_3^-] \cdot [H^+]}{[H_2CO_3]} = 10^{-6,035}\ mol\ L^{-1} \quad \text{(Ec.4.3)}$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \therefore K_{a2} = \frac{[CO_3^{2-}] \cdot [H^+]}{[HCO_3^-]} = 10^{-10,236}\ mol\ L^{-1} \quad \text{(Ec.4.4)}$$

Se ha llevado a cabo la determinación de la concentración de dióxido de carbono directamente en fase líquida mediante un electrodo específico, por lo que no es necesario aplicar la Ley de Henry. Para determinar la concentración total de dióxido de carbono disuelto, se considera la suma del CO_2 en fase líquida y el H_2CO_3, empleando el valor de K_M. Considerando los valores de K_{a1} y K_{a2}, se calculan las concentraciones de las otras dos especies presentes en el sistema ácido-base, obteniendo que, a la temperatura de trabajo (37 ºC) y el pH de trabajo (6,8 controlado), la distribución de las especies en el equilibrio es de un 25 % de CO_2 y de un 75 % de HCO_3^-, siendo despreciable la concentración de ion carbonato. Por lo tanto, la concentración de dióxido de carbono total disponible para el proceso será la suma de las tres especies (como se ha comentado, prácticamente de dos) que forman parte de los equilibrios ácido-base planteados ya que, al ir consumiendo dióxido de carbono, los equilibrios se irán desplazando para mantener las concentraciones de cada especie adecuadas.

Para este estudio se han llevado a cabo cuatro experimentos en biorreactor a agitaciones entre 100 a 700 rpm (empleando 20 g L^{-1} de xilosa como fuente de carbono y medio MPEL) en los que se cerró la entrada del citado gas al inocular el medio de cultivo. Debido a que se ha seguido la evolución del dióxido de carbono en fase líquida a lo largo de los experimentos de producción, aplicando los cálculos correspondientes a los diversos equilibrios comentados, se puede observar en la Figura 4.14, la evolución de todos los componentes del sistema ácido-base del CO_2, así como la suma de todos los compuestos para los cuarto experimentos realizados sin aporte de dióxido de carbono durante el proceso microbiano (100, 300, 500 y 700 rpm, Tabla 4.15). Asimismo, para poder determinar la influencia del aporte de dióxido de carbono al proceso estudiado de producción, en la Figura 4.15 se recogen también los resultados (como símbolos abiertos y líneas) de un experimento llevado a cabo a 300 rpm y con un aporte constante de dióxido de carbono con un caudal de 0,1 L min^{-1}. En la citada figura se puede observar cómo, durante aproximadamente las siete primeras horas de fermentación, no se observa consumo de dióxido de carbono y, posteriormente, al disminuir el dióxido de carbono disuelto en el caldo de cultivo debido al consumo realizado por las células, disminuyen

proporcionalmente el resto de las especies en el equilibrio. Como se puede observar, la disminución de las concentraciones de todos los componentes de este sistema ácido-base es mucho menos pronunciada en el caso del experimento en el que se aporta CO_2 de forma continua. La dispersión en los resultados se debe al error experimental y a las ligeras diferencias en los valores iniciales de dióxido de carbono en la fase líquida al comienzo de los diversos experimentos.

Tabla 4.15. Experimentos de influencia del aporte de CO_2 bajo distintas agitaciones.

Exp.	**55**	**56**	**57**	**58**	**59**
Agitación (rpm)	100	300	300	500	700
Corte caudal CO_2	Sí	Sí	No ($0{,}1$ L min^{-1})	Sí	Sí

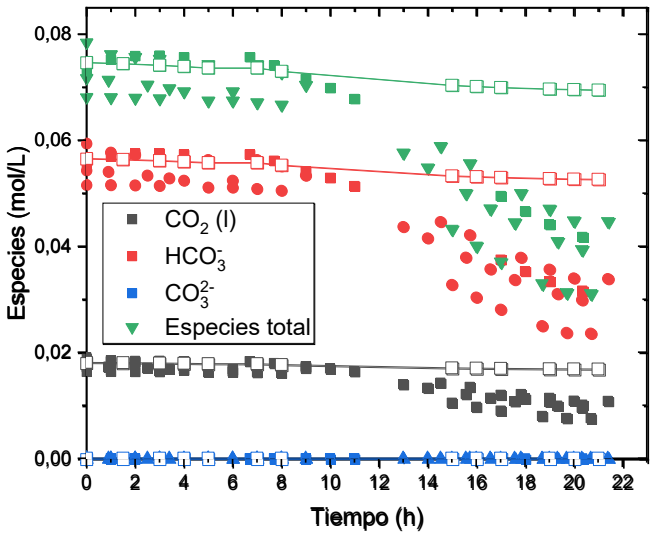

Figura 4.14. Evolución de todas las especies que forman parte del sistema de equilibrio ácido-base del dióxido de carbono, así como de la suma total de las mismas en los Exp. 55, 56, 57, 58, 59.

Para comparar la influencia de la transferencia de dióxido de carbono en el proceso, en la Figura 4.15 se recoge la evolución con el tiempo de biomasa y ácido succínico (Figura 4.15.A), y de fuente de carbono (xilosa) y subproductos (ácidos acético y fórmico) (Figura 4.15.B). En las citadas figuras se han vuelto a incluir los resultados del experimento con aporte de dióxido de carbono empleando símbolos abiertos.

En la Figura 4.15.A se puede comprobar que los resultados tanto de crecimiento como de producción de ácido succínico no se ven modificados ni por el empleo o no de adición continua de dióxido de carbono, ni por la agitación empleada para llevar el proceso. Asimismo, en la Figura 4.15.B, tampoco se observa influencia, como cabía esperar, ni en el consumo de la xilosa, ni en la producción de los subproductos, los ácidos acético y fórmico. Únicamente se podría destacar una menor producción de ácido acético en el experimento con aporte constante de dióxido de carbono.

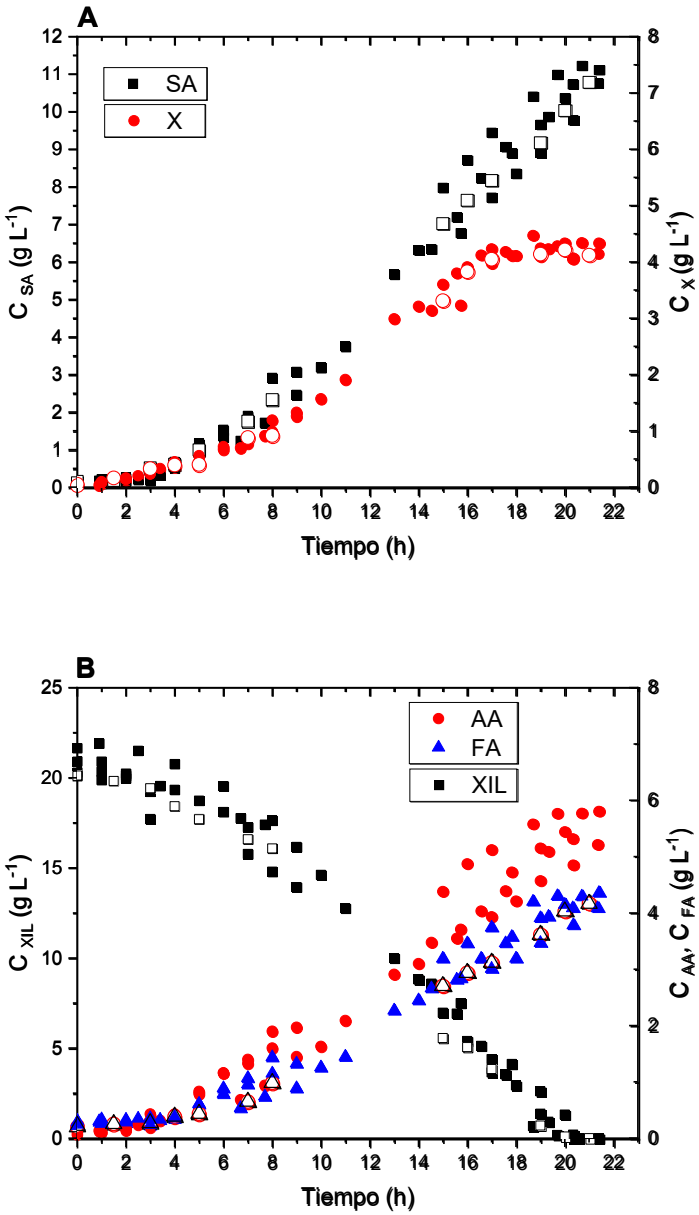

Figura 4.15. Evolución de la biomasa y del ácido succínico (A) y de la fuente de carbono (xilosa) y subproductos (ácidos acético y fórmico) (B) para los Exp. 55, 56, 57, 58, 59.

Estos resultados muestran que la velocidad de transferencia de dióxido de carbono en el sistema no es el fenómeno limitante de la velocidad global del proceso, lo que refuerza su no inclusión en el modelo cinético propuesto y aplicado en esta memoria.

4.7 MODELIZACIÓN CINÉTICA

Para poder llevar a cabo el escalado de un proceso de fermentación, es fundamental desarrollar modelos cinéticos con ecuaciones capaces de predecir el comportamiento de las especies involucradas a lo largo de la producción. Estos modelos permiten una adecuada selección del tipo de operación, así como el óptimo diseño y operación del biorreactor. Además, son de gran utilidad para la implementación del sistema de control y la realización de estudios tecno-económicos [71,181].

Sin embargo, hasta el momento, los modelos cinéticos desarrollados sobre la producción de ácido succínico se han basado exclusivamente en resultados de fermentaciones realizadas a diferentes concentraciones iniciales de sustrato, sin considerar los efectos de otras condiciones de operación de gran influencia. Además, la mayoría de estos modelos se limitan a la estimación de la velocidad de crecimiento de la biomasa, dejando de lado la evolución de los sustratos y productos del caldo [71]. Cabe destacar que algunos autores como Pateraki et al. [100], Lin et al. [98] y Vlysidis et al. [182] también se han centrado en la estimación de la concentración de la fuente de carbono, así como en la evolución del ácido succínico y los subproductos generados. Sin embargo, proponen sistemas de ecuaciones complejos con una elevada cantidad de parámetros de poco valor ingenieril.

4.7.1 Propuesta del modelo cinético para el empleo de células en crecimiento.

Se planteó un modelo cinético a partir de los experimentos de producción de ácido succínico a partir de glucosa pura y extracto de levadura comercial en *batch* presentados anteriormente en el apartado 4.2. En estas fermentaciones (Tabla 4.3), se modificaron distintas variables y condiciones de operación: concentración inicial de biomasa, de glucosa, de extracto de levadura, velocidad de agitación y caudal de CO_2 y se realizaron estimaciones de los parámetros cinéticos en todos los casos. Además, para comprobar la robustez del modelo, se llevó a cabo una fermentación tipo *fed-batch* (Exp.60) con el objetivo de realizar estimaciones en cada una de sus etapas y simular la evolución de las

concentraciones de las especies implicadas en la fermentación a partir de los intervalos de confianza de los parámetros cinéticos. Los resultados de estas estimaciones cinéticas junto a una discusión más pormenorizada se pueden encontrar en la **Publicación 3** "*Development of a Simple and Robust Kinetic Model for the Production of Succinic Acid from Glucose Depending on Different Operating Conditions*"

Se propuso un modelo no estructurado no segregado (el microorganismo se considera como un único componente, la biomasa) capaz de estimar la evolución de la concentración de biomasa, sustrato (glucosa) y productos de fermentación (ácidos succínico, acético y fórmico) del experimento de referencia (Exp. 9), cuya evolución a lo largo del tiempo se muestra en la Figura 4.16.A. Se observa que la biomasa crece hasta alcanzar su máximo a las 10 h de fermentación, sin embargo, tanto el ácido succínico como los subproductos continúan aumentando a lo largo del tiempo hasta agotar la fuente de carbono alrededor de las 33 h, lo que indica que la producción no está asociada al crecimiento. La velocidad de formación de ácido acético y fórmico se ralentiza después de aproximadamente 20 h de fermentación, mientras que la tasa de producción de ácido succínico sólo sufre una ligera reducción en las últimas horas del proceso. Además, aunque se produce una mayor cantidad de ácido acético que de ácido fórmico, ambos compuestos siguen la misma tendencia de crecimiento, por lo que se ha decidido agrupar ambos ácidos, como se muestra en la Figura 4.16.B, con el objetivo de proponer un modelo con el metabolito 'subproductos' (SP), logrando reducir el número de parámetros cinéticos y desarrollando un modelo más útil desde el punto de vista de la ingeniería química.

En base a estos datos, se ha propuesto un esquema de reacción muy simple (ecuaciones (4.5), 4.6) y (4.7)), compuesto por una primera reacción (r_1) de consumo de sustrato (S) para la generación de biomasa, una segunda reacción (r_2) de generación de ácido succínico (P) y SP y una última reacción (r_3) para la generación independiente de SP. Las velocidades de reacción (r_i) correspondientes se muestran en las ecuaciones (4.8), (4.9) y (4.10), mientras que las velocidades de consumo y formación (R_j) de los compuestos 'j' se describen en las ecuaciones (4.11), (4.12), (4.13) y (4.14). La biomasa tiene una velocidad de crecimiento basada en la ecuación logística, cuyos parámetros cinéticos son la velocidad específica de crecimiento (μ) y la concentración máxima de biomasa (C_{Xm}). Las velocidades de generación de ácido succínico y subproductos, no asociadas al crecimiento del microorganismo, se describen mediante ecuaciones de tipo

potencial. El modelo propuesto se ajustó a los datos experimentales de todos los experimentos realizados en reactor, obteniéndose los parámetros cinéticos y estadísticos que se muestran, respectivamente, en las Tablas 4.16 y 4.17. Por otra parte, en la Figura 4.16 B, se presenta, a modo de ejemplo, la buena reproducción de los datos experimentales obtenida del citado ajuste a los datos del experimento 9. Cabe señalar que, como se observa en la Tabla 4.15 por los valores de los parámetros estadísticos obtenidos de los ajustes, esta buena reproducción de los datos experimentales es general para todos los experimentos.

Esquema de reacción

$$Y_{S/X}S \xrightarrow{r_1} X \quad \text{(Ec.4.5)}$$

$$Y_{S/P1}S \xrightarrow{r_2} P + Y_{S/SP} \cdot SP \quad \text{(Ec.4.6)}$$

$$Y_{S/P2}S \xrightarrow{r_3} SP \quad \text{(Ec.4.7)}$$

Ecuaciones cinéticas

$$r_1 = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_{Xm}}\right) \quad \text{(Ec.4.8)}$$

$$r_2 = k_{P1} \cdot C_S \cdot C_X \quad \text{(Ec.4.9)}$$

$$r_3 = k_{P2} \cdot C_S \cdot C_X \quad \text{(Ec.4.10)}$$

Velocidades de consumo y producción

$$R_S = \frac{dC_S}{dt} = -Y_{S/X} \cdot r_1 - Y_{S/P1} \cdot r_2 - Y_{S/P2} \cdot r_3 \quad \text{(Ec.4.11)}$$

$$R_P = \frac{dC_P}{dt} = r_2 \quad \text{(Ec.4.12)}$$

$$R_X = \frac{dC_X}{dt} = r_1 \quad \text{(Ec.4.13)}$$

$$R_{SP} = \frac{dC_{SP}}{dt} = Y_{S/SP} \cdot r_2 + r_3 \quad \text{(Ec.4.14)}$$

Tabla 4.16. Parámetros cinéticos en función de las variables y condiciones de operación.

Exp.	Tipo Operación	C_X ($g \cdot L^{-1}$)	Agitación (rpm)	Caudal CO_2 ($L \cdot min^{-1}$)	C_{EL} ($g \cdot L^{-1}$)	C_S ($g \cdot L^{-1}$)	Cxm ± error ($g_X \cdot L^{-1}$)	K_{P1} ± error ($L \cdot g^{-1} \cdot h^{-1}$)	K_{P2} ± error ($L \cdot g^{-1} \cdot h^{-1}$)	μ ± error (h^{-1})
9	*Batch*	0,05	300	0,1	10	40	5,02 ± 0,02	0,007 ± 0,001	0,019 ± 0,001	0,85 ± 0,04
10	*Batch*	0,075	300	0,1	10	40	5,81 ± 0,03	0,009 ± 0,001	0,013 ± 0,001	0,85 ± 0,03
11	*Batch*	0,1	300	0,1	10	40	6,79 ± 0,08	0,004 ± 0,000	0,008 ± 0,001	0,85 ± 0,02
12	*Batch*	0,05	300	0,5	10	40	5,07 ± 0,03	0,008 ± 0,001	0,018 ± 0,001	0,85 ± 0,01
13	*Batch*	0,05	300	1	10	40	5,07 ± 0,03	0,008 ± 0,001	0,018 ± 0,001	0,85 ± 0,01
14	*Batch*	0,05	150	0,1	10	40	5,09 ± 0,09	0,004 ± 0,001	0,019 ± 0,001	0,85 ± 0,04
15	*Batch*	0,05	200	0,1	10	40	5,05 ± 0,06	0,006 ± 0,001	0,018 ± 0,001	0,85 ± 0,06
16	*Batch*	0,05	250	0,1	10	40	5,00 ± 0,05	0,008 ± 0,001	0,018 ± 0,001	0,85 ± 0,05
17	*Batch*	0,05	300	0,1	2,5	40	2,40 ± 0,02	0,008 ± 0,001	0,022 ± 0,001	0,85 ± 0,05
18	*Batch*	0,05	300	0,1	5	40	5,01 ± 0,03	0,004 ± 0,001	0,011 ± 0,001	0,85 ± 0,03
19	*Batch*	0,05	300	0,1	7,5	40	5,08 ± 0,03	0,005 ± 0,001	0,014 ± 0,001	0,85 ± 0,04
20	*Batch*	0,05	300	0,1	10	20	5,01 ± 0,02	0,028 ± 0,002	0,017 ± 0,002	0,85 ± 0,02
21	*Batch*	0,05	300	0,1	10	30	5,03 ± 0,03	0,013 ± 0,001	0,017 ± 0,004	0,85 ± 0,02
22	*Batch*	0,05	300	0,1	10	50	5,06 ± 0,05	0,003 ± 0,0001	0,018 ± 0,001	0,84 ± 0,03
60	*Fed-batch* ciclo 1	0,05	300	0,1	10	40	5,02 ± 0,02	0,007 ± 0,001	0,019 ± 0,001	0,85 ± 0,04
60	*Fed-batch* ciclo 2	0,05	300	0,1	10	40	5,02 ± 0,02	0,007 ± 0,001	0,019 ± 0,001	0,85 ± 0,04
60	*Fed-batch* ciclo 3	0,05	300	0,1	10	40	5,02 ± 0,02	0,004 ± 0,001	0,005 ± 0,000	0,85 ± 0,04

Tabla 4.16. Parámetros cinéticos en función de las variables y condiciones de operación (continuación).

Exp.	Tipo Op.	C_X ($g \cdot L^{-1}$)	Agit. (rpm)	Caudal CO_2 ($L \cdot min^{-1}$)	C_{EL} ($g \cdot L^{-1}$)	C_S ($g \cdot L^{-1}$)	$Y_{S/P1} \pm$ error ($g \cdot g^{-1}$)	$Y_{S/P2} \pm$ error ($g \cdot g^{-1}$)	$Y_{S/SP} \pm$ error ($g \cdot g^{-1}$)	$Y_{S/X} \pm$ error ($g \cdot g^{-1}$)
9	*Batch*	0,05	300	0,1	10	40	0,24 ± 0,02	2,27 ± 0,22	1,10 ± 0,09	1,65 ± 0,15
10	*Batch*	0,075	300	0,1	10	40	0,24 ± 0,02	2,20 ± 0,19	1,08 ± 0,07	1,63 ± 0,16
11	*Batch*	0,1	300	0,1	10	40	0,24 ± 0,02	2,25 ± 0,19	1,09 ± 0,08	1,63 ± 0,16
12	*Batch*	0,05	300	0,5	10	40	0,25 ± 0,02	2,27 ± 0,22	1,09 ± 0,09	1,66 ± 0,16
13	*Batch*	0,05	300	1	10	40	0,25 ± 0,02	2,27 ± 0,22	1,09 ± 0,09	1,66 ± 0,16
14	*Batch*	0,05	150	0,1	10	40	0,26 ± 0,02	2,30 ± 0,21	1,10 ± 0,08	1,56 ± 0,13
15	*Batch*	0,05	200	0,1	10	40	0,26 ± 0,02	2,27 ± 0,18	1,04 ± 0,07	1,54 ± 0,11
16	*Batch*	0,05	250	0,1	10	40	0,24 ± 0,02	2,31 ± 0,18	1,07 ± 0,09	1,60 ± 0,13
17	*Batch*	0,05	300	0,1	2,5	40	0,24 ± 0,02	1,45 ± 0,10	0,86 ± 0,01	0,98 ± 0,02
18	*Batch*	0,05	300	0,1	5	40	0,25 ± 0,01	1,76 ± 0,11	0,95 ± 0,02	1,65 ± 0,02
19	*Batch*	0,05	300	0,1	7,5	40	0,24 ± 0,01	2,06 ± 0,17	1,00 ± 0,04	1,65 ± 0,04
20	*Batch*	0,05	300	0,1	10	20	0,21 ± 0,023	2,26 ± 0,13	1,13 ± 0,14	1,67 ± 0,12
21	*Batch*	0,05	300	0,1	10	30	0,26 ± 0,022	2,27 ± 0,10	1,11 ± 0,11	1,63 ± 0,14
22	*Batch*	0,05	300	0,1	10	50	0,28 ± 0,020	2,26 ± 0,15	1,10 ± 0,09	1,63 ± 0,13
60	*Fed-batch* ciclo 1	0,05	300	0,1	10	40	0,24 ± 0,02	2,27 ± 0,22	1,10 ± 0,09	1,65 ± 0,15
60	*Fed-batch* ciclo 2	0,05	300	0,1	10	40	0,24 ± 0,02	2,27 ± 0,22	0,59 ± 0,02	1,65 ± 0,15
60	*Fed-batch* ciclo 3	0,05	300	0,1	10	40	0,24 ± 0,02	2,27 ± 0,22	0,37 ± 0,01	1,65 ± 0,15

Tabla 4.17. Parámetros estadísticos en función de las variables y condiciones de operación.

Exp.	Tipo operación	C_X (g L^{-1})	Agitación (rpm)	Caudal CO_2 (L min^{-1})	C_{EL} (g L^{-1})	C_S (g L^{-1})	F_{95}	RMSE	SSR	VE (%)
9	*Batch*	0,05	300	0,1	10	40	41242	0,67	6,47	99,5
10	*Batch*	0,075	300	0,1	10	40	13660	1,09	11,2	98,2
11	*Batch*	0,1	300	0,1	10	40	40640	1,00	10,0	98,7
12	*Batch*	0,05	300	0,5	10	40	8457	1,14	12,2	98,5
13	*Batch*	0,05	300	1	10	40	8457	1,14	12,2	98,5
14	*Batch*	0,05	150	0,1	10	40	19384	0,92	9,01	99,0
15	*Batch*	0,05	200	0,1	10	40	19684	1,03	9,99	98,6
16	*Batch*	0,05	250	0,1	10	40	11751	1,07	11,7	98,5
17	*Batch*	0,05	300	0,1	2,5	40	5037	1,19	14,3	97,3
18	*Batch*	0,05	300	0,1	5	40	22441	1,10	11,6	98,9
19	*Batch*	0,05	300	0,1	7,5	40	17270	1,00	6,3	98,9
20	*Batch*	0,05	300	0,1	10	20	30616	0,84	7,06	99,3
21	*Batch*	0,05	300	0,1	10	30	29587	0,87	8,76	98,6
22	*Batch*	0,05	300	0,1	10	50	33646	0,58	7,70	99,1
60	*Fed-batch* ciclo 1	0,05	300	0,1	10	40	41242	0,67	6,47	99,5
60	*Fed-batch* ciclo 2	0,05	300	0,1	10	40	13512	1,05	8,97	98,4
60	*Fed-batch* ciclo 3	0,05	300	0,1	10	40	24175	0,95	6,09	99,6

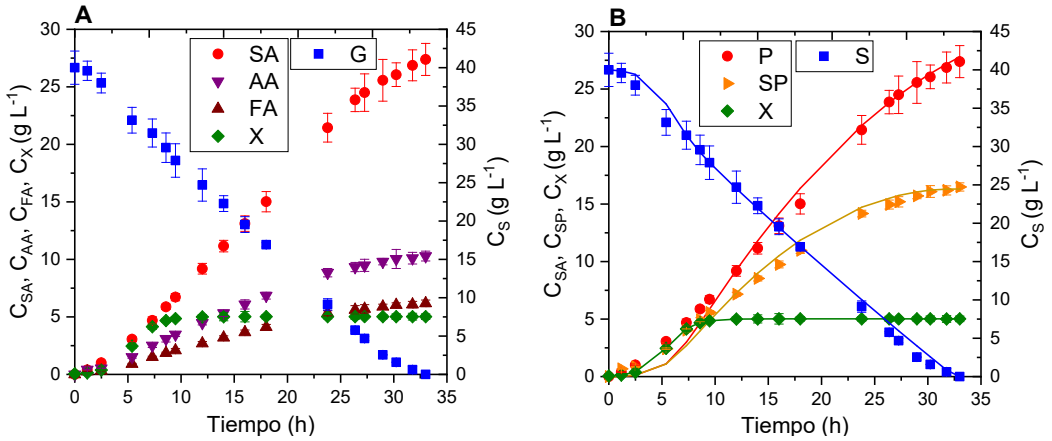

Figura 4.16. Evolución de las concentraciones de ácido succínico (SA) o producto (P), ácido acético (AA), ácido fórmico (FA), biomasa (X) y glucosa (G) o sustrato (S) a en el Exp.9 (referencia). Representación de las concentraciones de AA y FA por separado (A). *Lumping* de AA y FA en SP y modelización cinética (B).

La bondad del ajuste queda patente en los resultados de los parámetros estadísticos de la Tabla 4.15 (F de Fisher – 41242 - muy superior al valor tabulado al 95 % - 8,55, variación explicada (VE) – 99,5 % - próxima al 100 %, suma de residuos cuadráticos (SSR) - 6,47 - y error cuadrático medio residual (RMSE) - 0,67, próximos a cero), así como en el excelente ajuste a los valores experimentales que se puede observar en la Figura 4.16 (ajuste del modelo como líneas de la gráfica).

Al aplicar este modelo a los experimentos realizados a distintas **concentraciones iniciales de biomasa** (Exp. 9, 10, 11) se logró un buen ajuste a los datos experimentales, tal y como lo demuestran los parámetros estadísticos de la Tabla 4.17 y el bajo error de los intervalos de confianza de los parámetros cinéticos (Tabla 4.16). La mayoría de los parámetros cinéticos estimados no sufrieron modificaciones a distintos valores iniciales de concentración inicial de la biomasa, a excepción de C_{Xm}, k_{P1} y k_{P2}, cuyas variaciones se representan en la Figura 4.17.

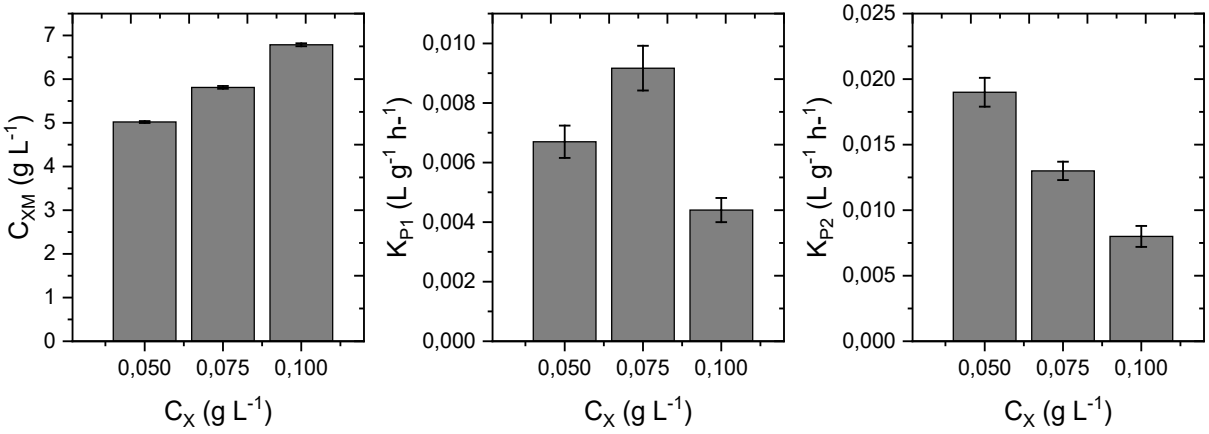

Figura 4.17. Parámetros cinéticos que se modifican en función de la concentración de biomasa inicial en los Exp.9, 10 y 11.

Por un lado, el valor del parámetro C_{Xm} revela que el aumento de la biomasa inicial también conduce a una mayor concentración máxima de biomasa en el caldo de cultivo una vez alcanzada la fase estacionaria de su crecimiento, con una tendencia prácticamente lineal, como se esperaba. Por otro lado, también se observa una disminución en la constante cinética de la reacción de formación del subproducto (k_{P2}), independiente de la del ácido succínico a medida que aumenta la densidad celular en el caldo, lo que concuerda con la tendencia al alza en la selectividad comentada anteriormente (apartado 4.2). Finalmente, se observa la correlación entre la productividad de ácido succínico (Tabla 4.3) y la constante cinética de la ecuación de formación de ácido succínico (k_{P1}), alcanzando su máximo a una concentración de biomasa inicial de 0,075 g L^{-1}.

Anteriormente (sección 4.2), se había observado que, al operar con **caudales de CO_2** comprendidos entre 0,1 y 1 L min^{-1}, intervalo que comprende los valores que se encuentran en bibliografía de bioproducción de ácido succínico, se está realizando la fermentación con exceso de este gas en todos los casos, pues no se observan diferencias significativas en los rendimientos y productividades. Esta deducción fue confirmada gracias a la aplicación simultánea del modelo cinético a los experimentos 9, 12 y 13, sin necesidad de variación de los parámetros cinéticos (Tabla 4.16) y con una adecuada bondad de ajuste (Tabla 4.17). La Figura 4.18 muestra la evolución de los datos experimentales de biomasa, sustrato, ácido succínico y subproductos a lo largo del tiempo en los experimentos 9, 12 y 13 junto con la representación de la estimación de la evolución de sus concentraciones realizada en conjunto.

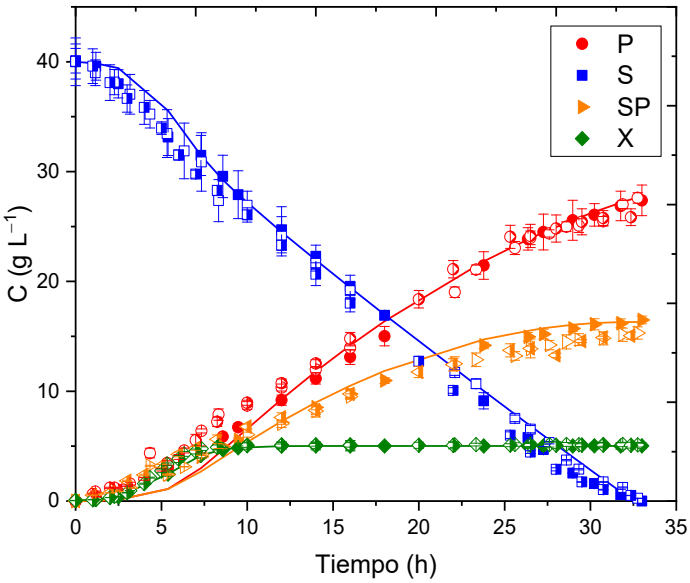

Figura 4.18. Modelo cinético de la evolución de las concentraciones de ácido succínico o producto (P), subproductos (SP), biomasa (X) y sustrato (S) a lo largo del tiempo en función del caudal de CO_2 en *batch* – Exp.9, 12, 13.

Al aplicar el modelo cinético a las fermentaciones realizadas a distintas **velocidades de agitación** (Exp. 9, 14, 15 y 16)**,** se observó en la constante cinética de la reacción para la formación de ácido succínico (k_{P1}) un crecimiento hasta alcanzar un máximo a 250 rpm y luego una reducción considerable a 300 rpm, como se muestra en la Figura 4.19. Esta respuesta de k_{P1} coincide con las tendencias comentadas anteriormente, observándose un máximo en el rendimiento y productividad del proceso a 250 rpm.

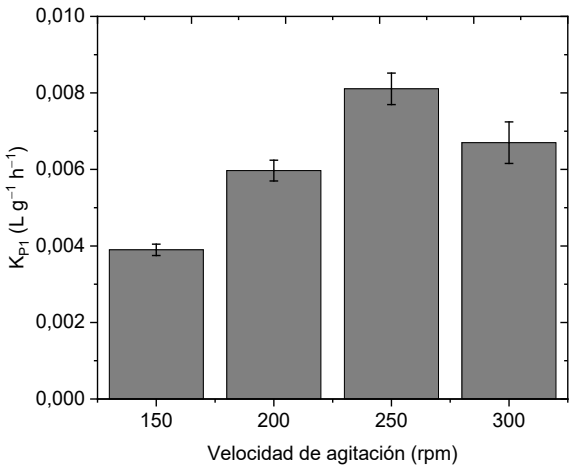

Figura 4.19. Parámetros cinéticos que se modifican en función de la velocidad de agitación en los Exp.9, 14, 15 y 16.

También se realizaron estimaciones de los parámetros cinéticos de las fermentaciones en las que se modificó la **concentración de la fuente de nitrógeno**, el extracto de levadura (EL). La Figura 4.20 muestra que las estimaciones cinéticas realizadas en los experimentos 9, 17, 18 y 19 dieron lugar a la variación de seis parámetros cinéticos. En primer lugar, en el experimento a 2,5 g L^{-1} de EL (Exp. 17) los valores de la concentración máxima de biomasa (C_{Xm}) y el rendimiento macroscópico de producción de biomasa ($Y_{S/X}$) fueron aproximadamente la mitad que los del correspondientes a todas las demás fermentaciones realizadas a concentraciones más altas de la fuente de nitrógeno (Exp. 9, 18, 19). Por lo tanto, teniendo en cuenta que no hay una gran disminución en el rendimiento entre el Exp. 17 y el resto de los experimentos, se puede deducir que la cantidad de succínico y subproductos generados por gramo de biomasa es mucho mayor a 2,5 g L^{-1} de EL que en los experimentos realizados a mayores concentraciones de EL, lo que se traduce en un aumento de los valores de k_{P1} y k_{P2}. También se puede observar que a partir de 5 g L^{-1} de EL, el aumento de la fuente de nitrógeno conduce a la misma evolución creciente de k_{P1} y k_{P2} que la productividad. A pesar de esto, se observa un aumento en los parámetros de rendimiento relacionados con los subproductos ($Y_{S/SP}$, $Y_{S/P2}$) a medida que aumenta la concentración inicial de EL, tanto en la reacción de generación simultánea de succínico y subproductos (r_2), así como en la reacción de generación de subproductos de manera aislada (r_3), lo cual concuerda con los datos presentados en la Tabla 4.3, es decir, con el aumento de la selectividad con la disminución de la cantidad inicial de EL.

Al llevar a cabo las estimaciones cinéticas de las fermentaciones a distintas **concentraciones iniciales de glucosa**, no se observaron variaciones estadísticamente significativas en función de las concentraciones iniciales de glucosa para la mayoría de los parámetros cinéticos estimados, excepto k_{P1} (Tabla 4.16). Esta constante cinética, como se muestra en la Figura 4.21, disminuye de manera claramente exponencial a medida que aumenta la concentración de sustrato, lo que confirma la inhibición del sustrato que se conjeturaba en el apartado 4.2. Debido a ello, se propuso una ecuación adicional en el modelo cinético, la ecuación (4.15), para el cálculo de este parámetro incorporando las constantes α y β, permitiendo el ajuste simultáneo de los experimentos a diferentes concentraciones iniciales de sustrato (Tabla 4.18).

$$k_{P1} = \alpha \cdot e^{-\beta \cdot C_{S0}} \qquad \text{(Ec.4.15)}$$

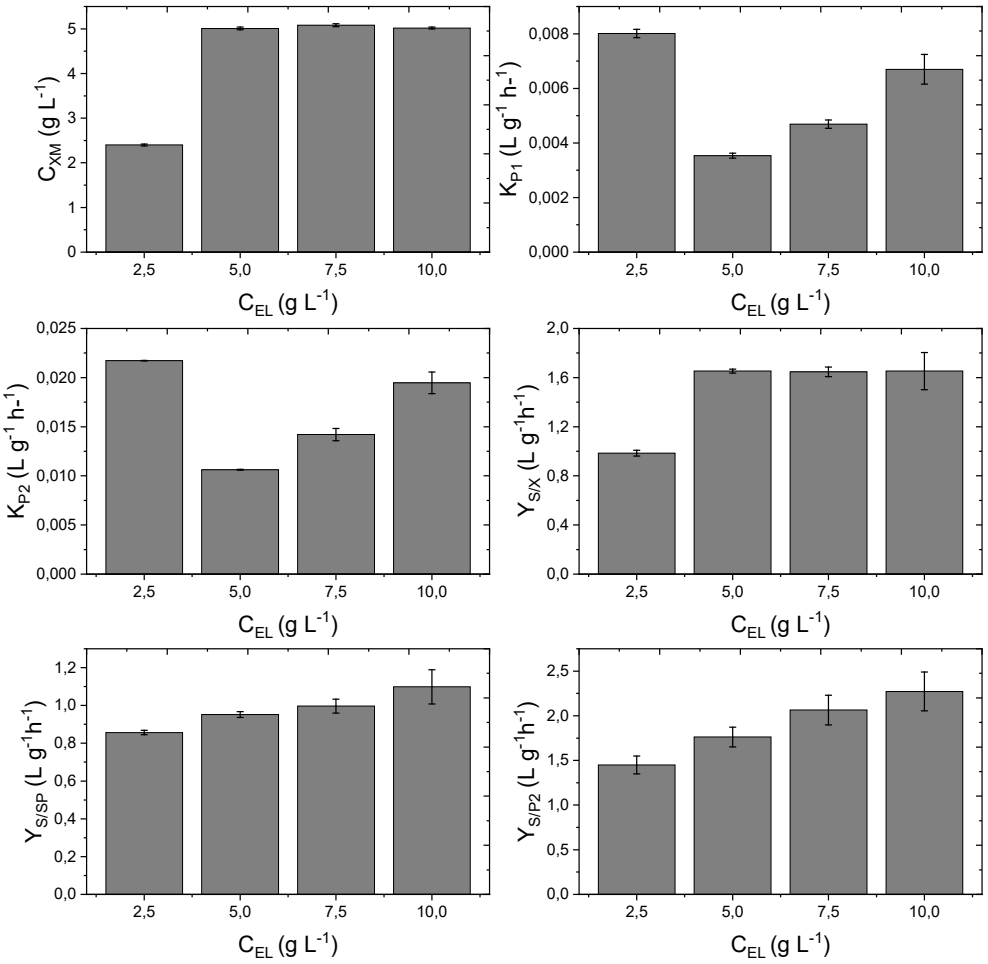

Figura 4.20. Parámetros cinéticos que se modifican en función de la concentración de extracto de levadura (EL) en los Exp.9, 17, 18 y 19.

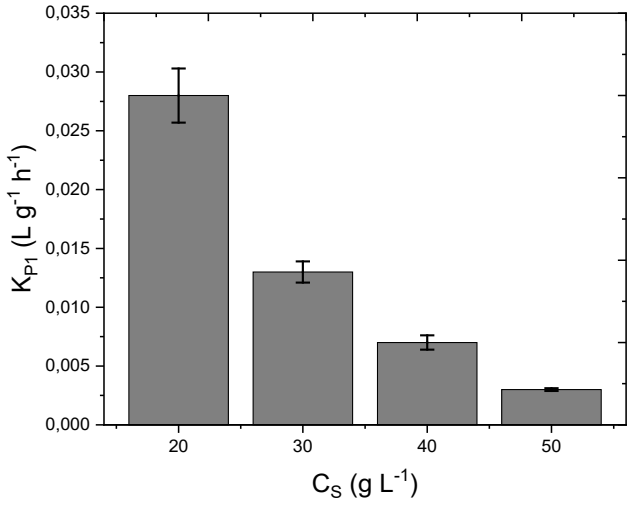

Figura 4.21. Parámetros cinéticos que se modifican en función de la concentración de sustrato en los Exp.9, 20, 21 y 22.

Tabla 4.18. Valores de parámetros cinéticos y estadísticos calculados ajustando el modelo cinético a datos experimentales de producción de ácido succínico a partir de diferentes concentraciones iniciales de sustrato simultáneamente.

C_{Xm} (g L^{-1})	5,095	±	0,027	**F_{95}**	20191
k_{P2} (L g^{-1} h^{-1})	0,017	±	0,001	**RMSE**	1,28
μ (h^{-1})	0,860	±	0,016	**SSR**	9,63
$Y_{S/P1}$ (g g^{-1})	0,240	±	0,066	**VE (%)**	97,8
$Y_{S/P2}$ (g g^{-1})	2,271	±	0,089		
$Y_{S/SP}$ (g g^{-1})	1,110	±	0,043		
$Y_{S/X}$ (g g^{-1})	1,620	±	0,087		
α (L·g^{-1}·h^{-1})	0,152	±	0,008		
β (L g^{-1})	0,079	±	0,001		

Con el objetivo de comprobar la robustez del modelo a tiempos de fermentación prolongados, se realizó una fermentación tipo ***fed-batch*** (Exp. 60), llevando a cabo ajustes del modelo cinético propuesto a cada una de las tres etapas y simulaciones de la evolución de la biomasa, sustrato, producto y subproductos en el tiempo a partir de los valores máximos y mínimos del intervalo de confianza de los parámetros cinéticos. La Figura 4.22 muestra los parámetros cinéticos que se ven modificados a lo largo de las tres etapas del proceso. La Figura 4.23 muestra las concentraciones experimentales de biomasa, sustrato y productos a lo largo del tiempo junto con las líneas de predicción del modelo cinético y las simuladas a partir del intervalo de confianza. También se han incluido los valores de rendimiento del ácido succínico respecto al sustrato consumido ($Y_{SA/S}$), así como la productividad y selectividad del ácido succínico en las tres etapas.

Cabe señalar que, en la segunda etapa, en consonancia con el aumento de la selectividad con respecto a la etapa anterior, se produce una disminución del parámetro de rendimiento hacia los subproductos ($Y_{S/SP}$) de la reacción 2. Por tanto, debido a una mayor utilización de sustrato en la producción de succínico en lugar de subproductos, es lógico que haya un aumento de rendimiento y productividad en esta etapa intermedia, sin necesidad de alterar ningún otro parámetro cinético del modelo. Por otro lado, debido al aumento de la selectividad de la tercera etapa con respecto a las anteriores, el comportamiento macroscópico hacia los subproductos de la reacción 2 ($Y_{S/SP}$) sufre una reducción considerable. Sin embargo, la caída en la productividad del ácido succínico solo se refleja en la disminución de las constantes cinéticas de las reacciones 2 y 3 (k_{P1} y k_{P2}), parámetros que disminuyen la velocidad de reacción de manera directamente proporcional. La simulación del primer ciclo del Exp. 60 se pudo realizar con los

parámetros estimados para Exp. 9 (experimento de referencia), ya que comparten las mismas condiciones de operación.

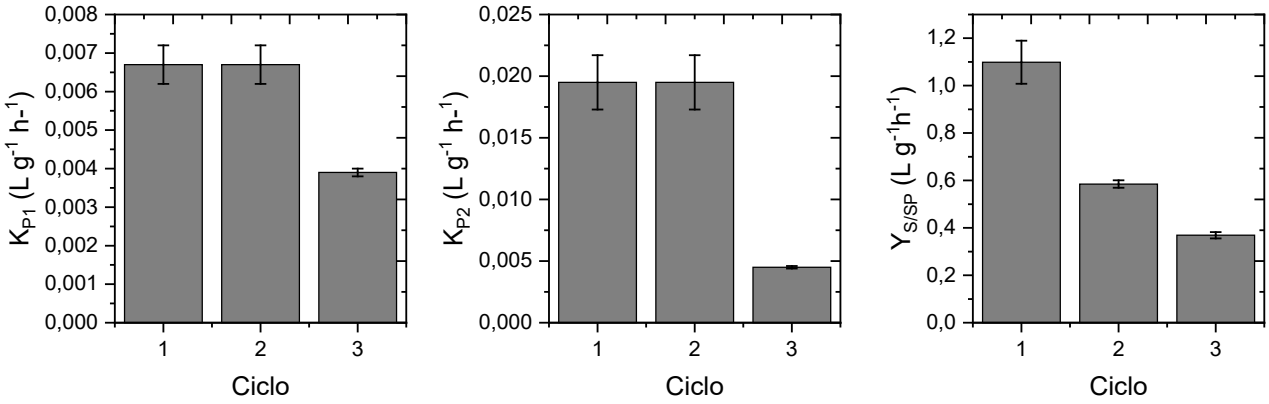

Figura 4.22. Parámetros cinéticos que se modifican en función del ciclo de la producción en operación tipo *fed-batch* – Exp. 60.

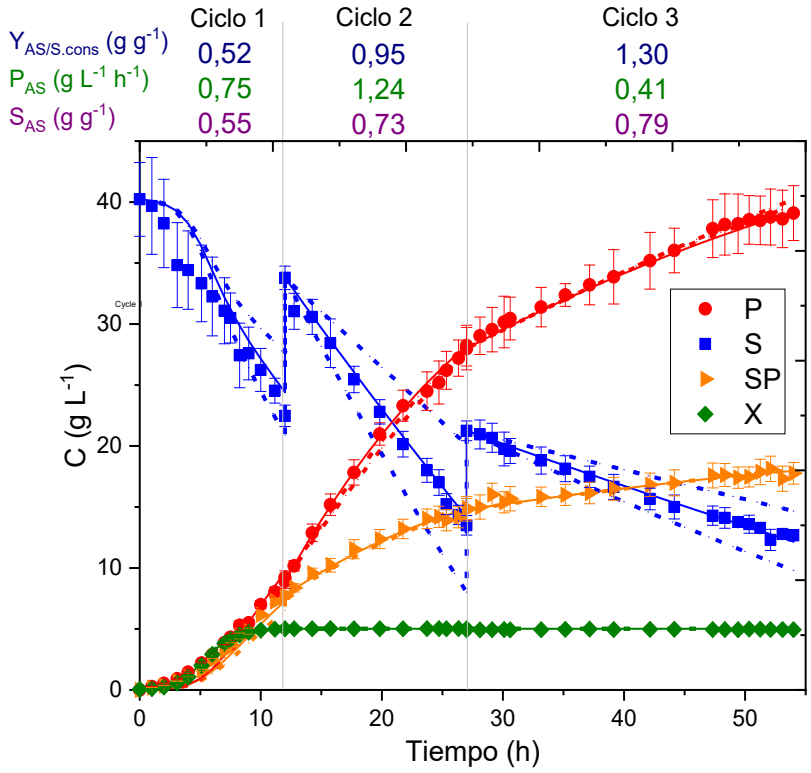

Figura 4.23. Evolución de la concentración de ácido succínico (SA), subproductos (SP), biomasa (X) y sustrato (S) a lo largo del tiempo en el Exp. 60 (*fed-batch*). $Y_{SA/S.cons}$: rendimiento en función del sustrato consumido, P_{SA}: productividad de ácido succínico, S_{SA}: selectividad.

4.7.2 Aplicación del modelo a las fermentaciones con hidrolizados de residuos de una cervecera

En el apartado 4.5.4, se demostró a viabilidad de la producción de ácido succínico sustituyendo simultáneamente residuos de una cervecera como fuente de nitrógeno (hidrolizado de levadura de cerveza gastada, HLCG) y como fuente de carbono (hidrolizado de bagazo de cerveza, HBC) – Exp.46. Con el objetivo de avanzar un paso más en el desarrollo de este proceso de cara a su escalado, se aplicó el modelo cinético desarrollado en el apartado 4.7.1 para glucosa pura y EL comercial a este experimento. Los parámetros cinéticos y estadísticos se muestran en la Tabla 4.19. El ajuste del modelo a los puntos experimentales de sustrato (*lumping* de azúcares), ácido succínico, subproductos (*lumping* de concentraciones de ácido acético y ácido fórmico) y biomasa se representa en la Figura 4.24.

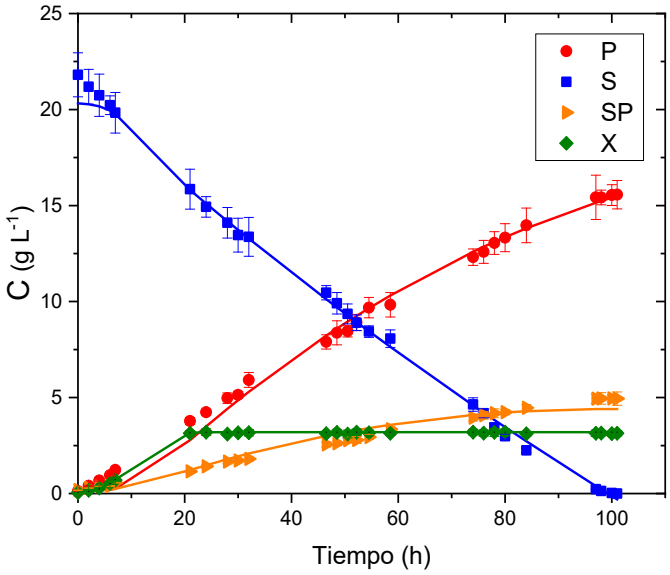

Figura 4.24. Modelado cinético de la producción de ácido succínico a partir de HBC como fuente de carbono y HLGC como fuente de nitrógeno en reactor tipo *batch* – Exp.46.

Tabla 4.19. Valores de parámetros estadísticos y cinéticos calculados ajustando el modelo cinético a datos experimentales de producción de ácido succínico en *batch* con HBC como fuente de carbono y HLGC como fuente de nitrógeno.

C_{Xm} (g L^{-1})	3,20	±	0,07	**F_{95}**	8016
kp_1 10^{-3} (L h g^{-1})	3,06	±	0,21	**RMSE**	0,36
kp_2 10^{-2} (L h g^{-1})	8,52	±	0,07	**SSR**	6,52
μ (h^{-1})	0,41	±	0,01	**VE (%)**	99,4
$Y_{S/P1}$ (g g^{-1})	0,39	±	0,02		
$Y_{S/P2}$ (g g^{-1})	1,99	±	0,21		
$Y_{S/SP}$ (g g^{-1})	0,55	±	0,04		
$Y_{S/X}$ (g g^{-1})	0,73	±	0,03		

Se puede en la Figura 4.24 que el ajuste del modelo a los datos experimentales es muy preciso (líneas en la Figura 4.24). Asimismo, en la Tabla 4.19 se observa que, tanto el bajo intervalo de confianza que presentan los valores de los parámetros cinéticos, así como los valores estadísticos (elevados valores de la F de Fisher, VE próximo al 100% y bajos resultados de RMSE y SSR) demuestran la bondad del ajuste.

4.7.3 Aplicación del modelo cinético a fermentaciones con hidrolizados de residuos de patata

Tras haber realizado con éxito una fermentación en *batch* a partir de un hidrolizado de residuos de patata (HRP) en el apartado 4.5.7, de nuevo se quiso comprobar la viabilidad de la aplicación del modelo cinético desarrollado en el apartado 4.2 en otro proceso en el que se emplean residuos en vez de azúcares puros. Se recurrió a este modelo ampliado con la ecuación (4.15), desarrollada para el cálculo de k_{P1} en función de la concentración inicial de glucosa. En la Figura 4.25 se presenta la evolución del sustrato, ácido succínico, subproductos (ácidos acético y fórmico) y la biomasa en el experimento de producción de ácido succínico mediante fermentación en reactor de HRP junto al ajuste (en líneas continuas) del modelo cinético a los datos experimentales. En la Tabla 4.18 se muestran los parámetros cinéticos y estadísticos obtenidos en el ajuste.

La bondad del modelo no solo queda justificada por la precisión del ajuste de las líneas del modelo a los puntos experimentales, sino por los bajos valores de RMSE y elevados resultados de F (siendo más elevados que los necesarios para superar la hipótesis nula al 95 % de confianza, intervalo del valor F umbral tabulado al 95 % de confianza para los grados de libertad relevantes del numerador y el denominador: 20- 30). Además,

los valores de VE son próximos al 100 %, por lo que el modelo cinético explica fácilmente la evolución de todas las variables dependientes del tiempo del proceso.

Tabla 4.20. Valores de parámetros cinéticos y estadísticos calculados ajustando el modelo cinético a datos experimentales de producción de ácido succínico a partir de HRP en *batch*.

C_{Xm} (g L^{-1})	6,587	±	0,043	**F_{95}**	30473
kp_2 (L g^{-1} h^{-1})	0,007	±	0,0003	**RMSE**	0,88
μ (h^{-1})	0,878	±	0,042	**SSR**	6,18
$Y_{S/P1}$ (g g^{-1})	1,337	±	0,066	**VE (%)**	98,4
$Y_{S/P2}$ (g g^{-1})	0,585	±	0,045		
$Y_{S/BP}$ (g g^{-1})	0,363	±	0,018		
$Y_{S/X}$ (g g^{-1})	0,415	±	0,015		
α (L g^{-1} h^{-1})	0,157	±	0,001		
β (L g^{-1})	0,073	±	0,001		

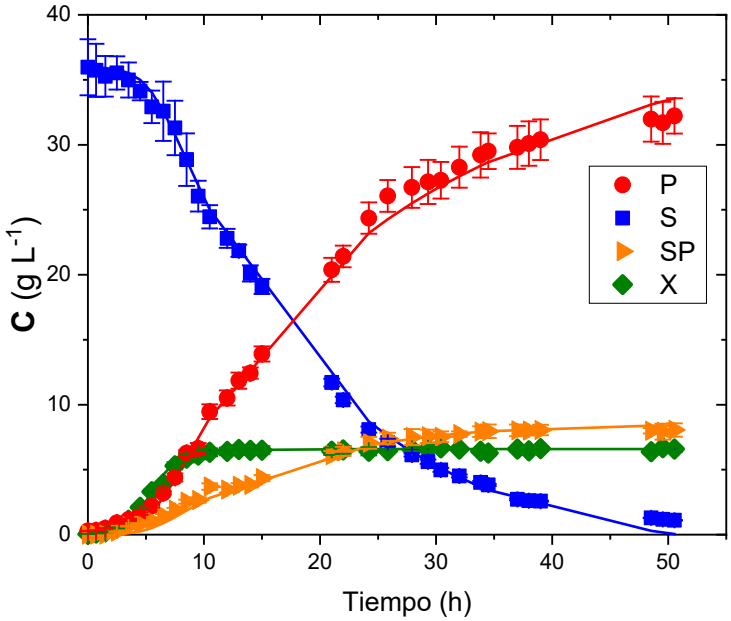

Figura 4.25. Modelado cinético de la producción de ácido succínico a partir de HRP en *batch* – Exp-50.

Comparando los valores de los parámetros cinéticos obtenidos con glucosa pura (Exp. 49) y con HRP (Exp. 50), se puede apreciar que existe una mayor concentración de biomasa final (C_{Xm}) (6,587 g L^{-1} vs. 5,020 g L^{-1}) al utilizar el HRP, pero, al mismo tiempo, apreciamos una menor velocidad de crecimiento de la misma (μ es de 0,878 h^{-1} mientras que con glucosa pura es de 0,923 h^{-1}), probablemente debido a la presencia de sustancias

tóxicas como el 5-HMF o los compuestos fenólicos presentes en las patatas, así como en las aguas y soluciones derivadas de su procesamiento [183]. El aumento del rendimiento de ácido succínico queda reflejado en el aumento de $Y_{S/P1}$ (1,337 g g^{-1}, mientras que con glucosa pura fue 0,240 g g^{-1}). La disminución de $Y_{S/BP}$ e $Y_{S/P2}$ justifica el incremento de la selectividad siendo, respectivamente, 1,102 g g^{-1} y 2,271 g g^{-1} con glucosa pura y 0,363 g g^{-1} y 0,585 g g^{-1} con el hidrolizado. Además, k_{P2} también disminuye de 0,019 L g^{-1} h^{-1} con glucosa comercial a 0,007 L g^{-1} h^{-1} con el hidrolizado.

4.7.4 Aplicación del modelo a fermentaciones tipo *repeated batch* con hidrolizados de residuos de patata y de levadura de cerveza gastada.

Finalmente, se quiso comprobar la viabilidad de la aplicación de este modelo cinético en las producciones en modo *repeated batch* con glucosa o HRP y EL o HLGC (Exp. 51 – 54), experimentos anteriormente expuestos en el apartado 4.5.8. Los parámetros cinéticos y estadísticos estimados se muestran en las Tablas 4.21 y 4.22, respectivamente. En la Figura 4.26 se representan los parámetros cinéticos que sufren variaciones a lo largo de los ciclos de fermentación y en la Figura 4.27 se muestra el ajuste del modelo cinético a los datos experimentales de concentración de las especies implicadas en la fermentación a lo largo del tiempo de los Exp. 51, 52, 53 y 54.

Los parámetros que se vieron modificados entre las etapas fueron las constantes cinéticas k_{P1} y k_{P2} y los parámetros asociados al crecimiento de biomasa (C_{Xm} y μ). Independientemente de la fuente de carbono o nitrógeno utilizada, C_{Xm} aumentó a lo largo de las tres etapas de los procesos. Sin embargo, en todos los casos, μ fue mucho mayor en la primera etapa en relación a las demás, siendo esta caída especialmente pronunciada en los experimentos realizados con glucosa pura como fuente de carbono y HLCG como fuente de nitrógeno. k_{P1} siguió tendencias similares a las productividades de las etapas observadas en la Tabla 4.14. En los experimentos en los que se utilizó EL como fuente de nitrógeno, la máxima productividad se alcanzó en la tercera etapa, al igual de k_{P1}, mientras que, en los casos en que se utilizó HLCG como nutriente, la máxima productividad se obtuvo en la segunda etapa, observándose una ligera disminución de k_{P1} en el tercer ciclo. En cuanto a k_{P2}, se observa una tendencia creciente en todos los casos debido a que, en todos ellos, la selectividad resultó perjudicada por la adición de etapas

en el proceso. Este aumento en la generación de subproductos y, por tanto, de k_{P2}, fue especialmente acentuado en los casos en los que se utilizó EL como fuente de nitrógeno.

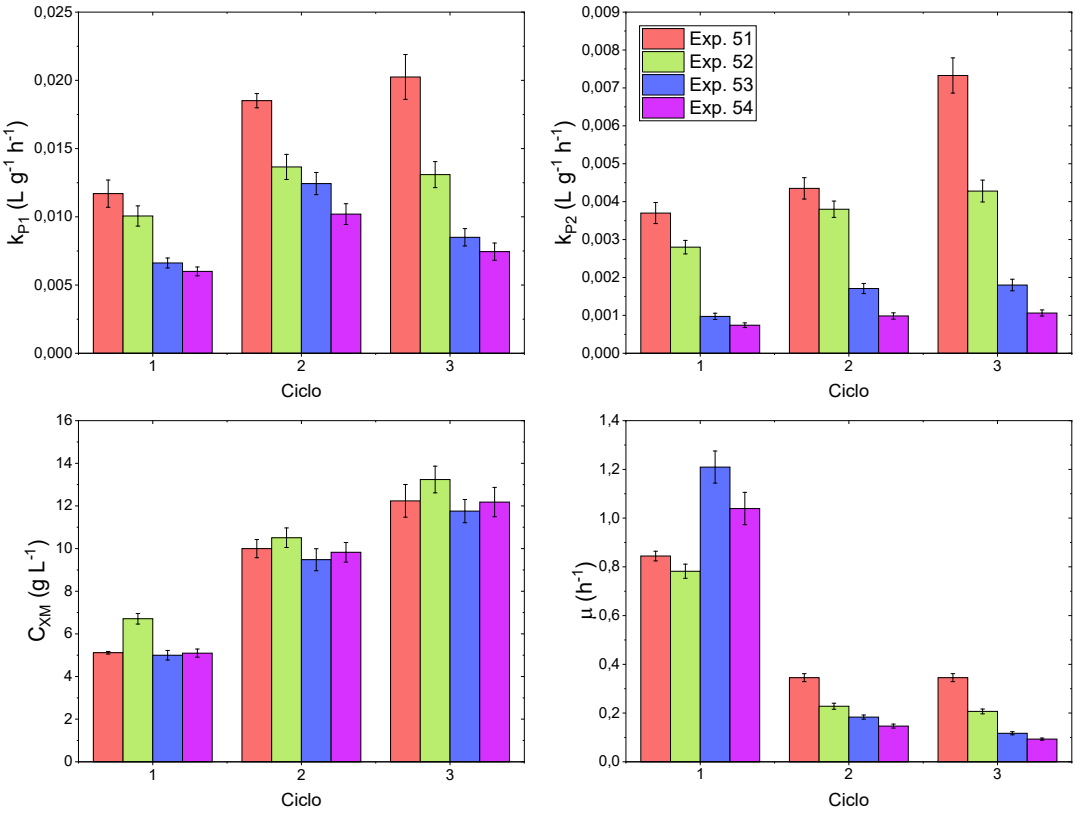

Figura 4.26. Parámetros cinéticos que sufren modificaciones durante las tres etapas de producción en modo *repeated batch* a partir de hidrolizados de patata (HRP) o glucosa y levadura de cerveza gastada (HLCG) o EL.

Tabla 4.21. Parámetros cinéticos de la fermentación tipo *repeated batch* a partir de hidrolizados de patata (HRP) o glucosa y levadura de cerveza gastada (HLCG) o EL.

	FC: GLUCOSA			FC: HRP			FN: EL			FN: HLGC		
Ciclo	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Exp.		**51**			**52**			**53**			**54**	
C_{Xm} **(g L^{-1})**	5,12 ± 0,06	10,1 ± 0,43	12,2 ± 0,77	6,71 ± 0,25	1,05 ± 0,05	1,32 ± 0,06	5,00 ± 0,23	9,48 ± 0,52	1,18 ± 0,05	5,10 ± 0,19	9,83 ± 0,46	1,22 ± 0,07
k_{P1} **(L g^{-1} h^{-1})**	(1,17 ± 0,09)·10^{-2}	(1,85 ± 0,05) ·10^{-2}	(2,02 ± 0,16)·10^{-2}	(1,01 ± 0,07)·10^{-2}	(1,37 ± 0,09) · 10^{-2}	(1,31 ± 0,0)	(6,62 ± 0,36)·10^{-3}	(1,24 ± 0,08) ·10^{-2}	(8,50 ± 0,63) ·10^{-3}	(6,00 ± 0,32) ·10^{-3}	(1,02 ± 0,08) ·10^{-2}	(7,45 ± 0,63) ·10^{-3}
k_{P2} **(L g^{-1} h^{-1})**	(3,70 ± 0,28) ·10^{-3}	(4,35 ± 0,28)·10^{-3}	(7,33 ± 0,46)·10^{-3}	(2,80 ± 0,18)·10^{-3}	(3,80 ± 0,21) · 10^{-3}	(4,28 ± 0,28) · 10^{-3}	(9,73 ± 0,82) ·10^{-4}	(1,71 ± 0,13) ·10^{-3}	(1,80 ± 0,15) ·10^{-3}	(7,41 ± 0,62) ·10^{-4}	(9,84 ± 0,08) ·10^{-4}	(1,06 ± 0,08) ·10^{-3}
μ (h^{-1})	(8,45 ± 0,19) ·10^{-1}	(3,45 ± 0,16) ·10^{-1}	(3,45 ± 0,16)·10^{-1}	(7,82 ± 0,28)·10^{-1}	(2,28 ± 0,12) · 10^{-1}	(2,07 ± 0,09) · 10^{-1}	1,21 ± 0,06	1,21 ± 0,09	(1,17 ± 0,06) ·10^{-1}	1,04 ± 0,06	(1,47 ± 0,08) ·10^{-1}	(9,32 ± 0,04) ·10^{-2}
$Y_{S/P1}$ **(g g^{-1})**	(6,62 ± 0,51)· 10^{-1}	(6,62 ± 0,51)· 10^{-1}	(6,62 ± 0,51)· 10^{-1}	(9,68 ± 0,42) · 10^{-1}	(9,68 ± 0,42) · 10^{-1}	(9,68 ± 0,42) · 10^{-1}	(8,62 ± 0,56) ·10^{-1}	(8,62 ± 0,56) ·10^{-1}	(8,62 ± 0,56) ·10^{-1}	(7,86 ± 0,29) ·10^{-1}	(7,86 ± 0,29) ·10^{-1}	(7,86 ± 0,29) ·10^{-1}
$Y_{S/P2}$ **(g g^{-1})**	2,20 ± 0,18	2,20 ± 0,18	2,20 ± 0,18	(2,43 ± 0,20) · 10^{-1}	(2,43 ± 0,20) · 10^{-1}	(2,43 ± 0,20) · 10^{-1}	1,21 ± 0,09	1,21 ± 0,09	1,21 ± 0,09	1,87 ± 0,08	1,87 ± 0,08	1,87 ± 0,08
$Y_{S/SP}$ **(g g^{-1})**	(3,29 ± 0,21) · 10^{-1}	(3,29 ± 0,21) · 10^{-1}	(3,29 ± 0,21) · 10^{-1}	(8,41 ± 0,62) · 10^{-3}	(8,41 ± 0,62) · 10^{-3}	(8,41 ± 0,62) · 10^{-3}	(1,36 ± 0,12) ·10^{-1}	(1,36 ± 0,12) ·10^{-1}	(1,36 ± 0,12) ·10^{-1}	(1,32 ± 0,07) ·10^{-1}	(1,32 ± 0,07) ·10^{-1}	(1,32 ± 0,07) ·10^{-1}
$Y_{S/X}$ **(g g^{-1})**	(2,89 ± 0,19) · 10^{-1}	(2,89 ± 0,19) · 10^{-1}	(2,89 ± 0,19) · 10^{-1}	(4,17 ± 0,27) · 10^{-1}	(4,17 ± 0,27) · 10^{-1}	(4,17 ± 0,27) · 10^{-1}	(2,61 ± 0,17) ·10^{-1}	(2,61 ± 0,17) ·10^{-1}	(2,61 ± 0,17) ·10^{-1}	(2,01 ± 0,13) ·10^{-1}	(2,01 ± 0,13) ·10^{-1}	(2,01 ± 0,13) ·10^{-1}

En la Tabla 4.22 y la Figura 4.27 se puede apreciar cómo el modelo se ajusta con mucha precisión a los datos experimentales en todas las etapas de los procesos llevados a cabo en modo *repeated batch,* independientemente de la fuente de carbono o de nitrógeno empleada. Los valores de RMSE (0,81 – 2,07) y SSR (11,42 – 21,80) son bajos, mientras que los valores VE (94,9 – 99,0 %) están cerca del 100 % y los valores F de Fisher (1080 – 7648) son mucho más altos que los necesarios para superar la hipótesis nula al 95 % (8,55 es el valor de la F tabulado a esa probabilidad).

Tabla 4.22. Parámetros estadísticos obtenidos en el ajuste del modelo cinético a los datos experimentales de producción de ácido succínico en modo *repeated batch* en función de la fuente de carbono (FC) (glucosa o HRP) y la fuente de nitrógeno (FN) (EL o HLGC).

	FC: GLUCOSA			**FC: HRP**			
Ciclo	**1**	**2**	**3**	**1**	**2**	**3**	
Exp.		**51**			**52**		**FN: EL**
F	5238	3358	1967	2842	4959	1080	
RMSE	1,07	1,14	1,30	0,86	0,98	2,07	
SSR	15,05	14,64	14,09	14,11	13,83	21,55	
VE (%)	98,7	97,7	98,6	99,0	98,4	94,9	
Exp.		**53**			**54**		**FN: HLCG**
F	1695	7648	1518	1853	5313	2264	
RMSE	1,37	0,81	1,97	1,41	1,08	1,86	
SSR	11,42	12,39	21,56	21,74	14,65	21,80	
VE (%)	97,3	99,3	96,9	97,3	98,7	97,2	

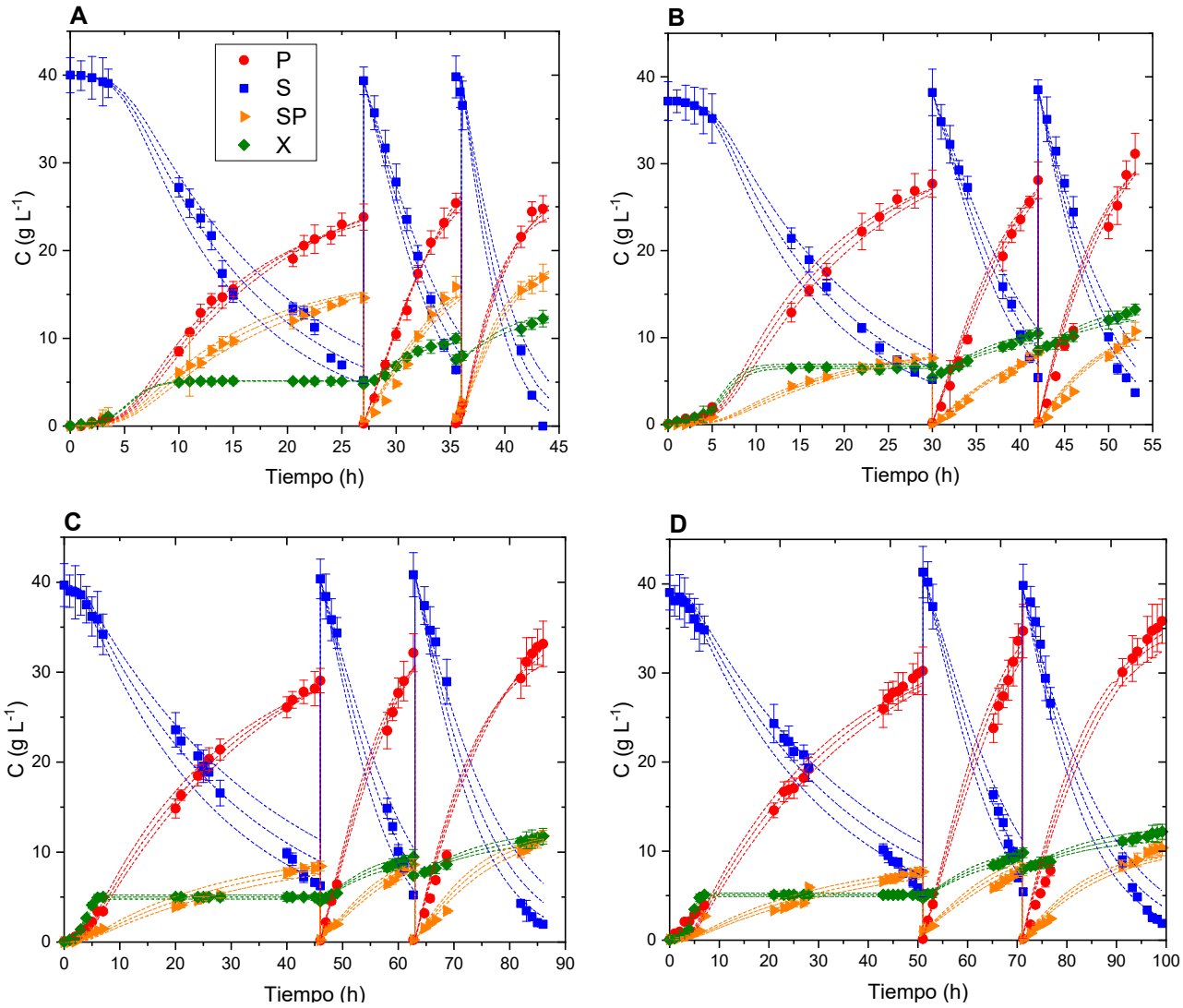

Figura 4.27. Evolución de las concentraciones del sustrato, ácido succínico, subproductos (ácidos acético y fórmico) y biomasa (X) en *repeated batch*. Exp.51 (gráfico A), Exp.52 (gráfico B), Exp.53 (gráfico C), Exp.54 (gráfico D).

4.7.5 Modelo cinético con células en estado de *resting.*

En el apartado 4.3 se había estudiado la producción con células en estado de *resting*, determinando, en primer lugar, la necesidad de adaptar a la fuente de carbono y realizar dos etapas de inóculo de las células en crecimiento en medio TSB para poder alcanzar rendimientos competitivos con *resting cells* (apartado 4.3.1). También se observó que los mejores resultados se alcanzaban al emplear como inóculo células en suspensión en el caldo de cultivo tras 15 h de crecimiento (apartado 4.3.2). El experimento llevado a cabo bajo todas estas condiciones consideradas óptimas fue el Exp. 26. Por ello, se quiso plantear un modelo cinético sencillo, no segregado - no estructurado, para la

predicción de la evolución de la concentración de sustrato (S), en este caso xilosa, producto (P) y subproductos (SP, ácidos acético y fórmico).

Dado que, durante una fermentación en ausencia de una fuente de nitrógeno, se detiene la reproducción celular, el esquema de reacción química que describe la producción de ácido succínico y subproductos puede llegar a reducirse a una sola expresión. En las ecuaciones (4.16) - (4.20) se muestra la reacción de producción de ácido succínico y subproductos, así como la velocidad de reacción (r_1) y las velocidades de formación de producto (R_P) y subproductos (R_{SP}) y consumo de sustrato (R_S). Todas las velocidades son de tipo potencial. A pesar de la simplicidad del modelo, se logra un buen ajuste a los datos experimentales (Tabla 4.23), tal y como lo demuestra un alto valor de la F de Fisher (el valor de la F tabulado al 95% de probabilidad es 8,55), de la variación explicada (VE) y valores de RMSE y SSR próximos a cero. En la Figura 4.28 se plasma el ajuste del modelo planteado a la evolución a lo largo del tiempo de las concentraciones del sustrato y productos.

Esquema de reacción	$Y_{S/P} \cdot S \rightarrow P + Y_{SP/P} \cdot SP$	(Ec.4.16)
Ecuación cinética	$r_1 = k_p \cdot C_S \cdot C_X$	(Ec. 4.17)
Velocidades de consumo y producción	$R_S = -Y_{S/P} \cdot r_1$	(Ec. 4.18)
	$R_P = r_1$	(Ec. 4.19)
	$R_{SP} = Y_{SP/P} \cdot r_1$	(Ec. 4.20)

Tabla 4.23. Valores de parámetros cinéticos y estadísticos calculados ajustando el modelo cinético a datos experimentales de producción de ácido succínico a partir de células en *resting* libres adaptadas a la fuente de carbono, con etapa de preinóculo, tras 15 h de crecimiento – Exp.26.

K_p x 10^{-3} (h L g^{-1})	3,12 ± 0,05	**F_{95}**	1320
$Y_{S/P}$ (g g^{-1})	1,27 ± 0,02	**RMSE**	0,48
$Y_{SP/P}$ (g g^{-1})	0,35 ± 0,02	**SSR**	27,4
		VE (%)	94,9

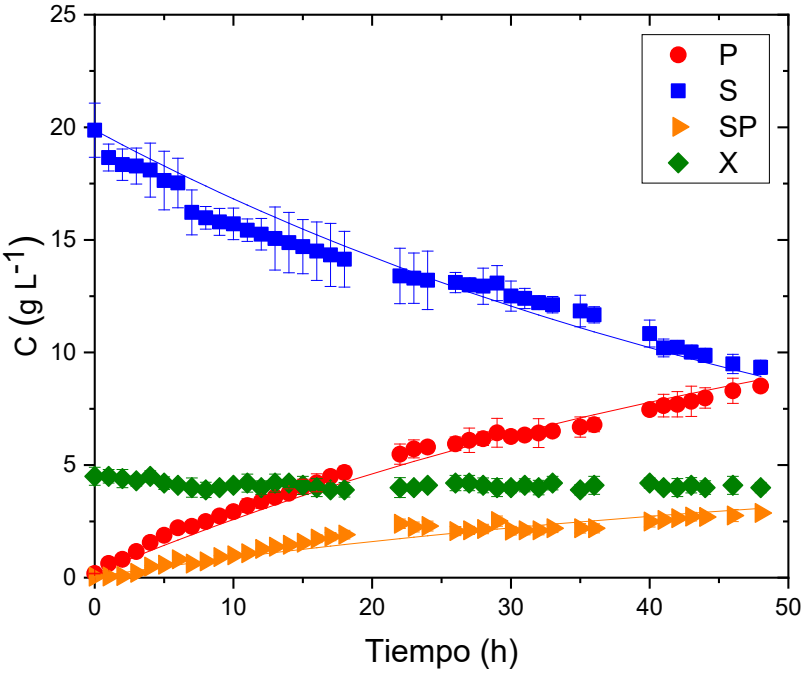

Figura 4.28. Modelado cinético de la producción de ácido succínico con células en *resting* libres adaptadas a la fuente de carbono, con etapa de preinóculo, tras 15 h de crecimiento –Exp.26.

4.7.6 Aplicación del modelo cinético a fermentaciones tipo *repeated batch* y *fed-batch* con medio MPEL: Comparación con células en crecimiento

En el apartado 4.2 se comparó la operación tipo *fed-batch* y *repeated batch* tanto con células en crecimiento como en estado de *resting*. En estos experimentos se empleó como medio de crecimiento celular MPEL, tras haberse comprobado su mejor rendimiento y menores costes que TSB (apartado 4.3.4).

Para modelizar las operaciones *fed-batch* y *repeated batch* con *resting cells* y células en crecimiento, se planteó una variación del modelo del apartado 4.7.7, basándose de nuevo en una única reacción de producción de productos y subproductos, pero incorporando el término de la biomasa (Ec. 4.21), de modo que este modelo sirviera tanto para los experimentos con células en crecimiento como para células en *resting* (igualando a cero los términos asociados al crecimiento de la biomasa). Para este modelo se conservaron las ecuaciones 4.17, 4.18, 4.19 y 4.20 del apartado 4.3.3 y se incorporaron las ecuaciones 4.21 y 4.22.

$$Y_{S/P}\ S \xrightarrow{r} Y_{X/P}\ X + P + Y_{SP/P}\ SP \quad \text{(Ec.4.21)}$$

$$R_X = \frac{d[X]}{dt} = Y_{X/P} \cdot r \quad \text{(Ec.4.22)}$$

En las Tablas 4.24 y 4.25 se presentan los parámetros cinéticos y estadísticos obtenidos en el ajuste a los datos experimentales con el modelo planteado en fermentaciones tipo *fed-batch* y *repeated batch*, tanto con células en estado de *resting* como de crecimiento. En la Figura 4.29 se representan los parámetros cinéticos que sufren variaciones a lo largo de los ciclos y en la Figura 4.30 el ajuste del modelo cinético a los datos experimentales.

El parámetro cinético k_P sufre una disminución a lo largo de los ciclos de reutilización, independientemente del estado del biocatalizador y del modo de operación. Los parámetros cinéticos concuerdan con las observaciones de desactivación del biocatalizador a lo largo de los ciclos de fermentación. Sin embargo, la desactivación de las células en crecimiento en *repeated batch* tiene lugar más lentamente que en las otras condiciones. En cuanto a los rendimientos (sustrato del producto, subproducto del producto y biomasa del producto), los valores más pequeños indican un bioproceso de producción de SA efectivo, ya que se requiere menos sustrato y biomasa para la generación del producto con una menor generación de productos secundarios. Como se refleja en las tendencias de rendimiento, el biocatalizador compuesto por células en *resting* es más específico para la producción de SA tipo *fed-batch*, especialmente en el segundo y tercer ciclo.

Los parámetros estadísticos de bondad de ajuste indican un valor alto para la F de Fisher, muy por encima del valor límite, y un valor bajo para el RMSE y el SSR. Además, las tendencias experimentales (Figura 4.30) y las predichas por el modelo son bastante similares, como muestran los porcentajes de VE superiores al 90%.

Tabla 4.24. Valores de parámetros estadísticos y cinéticos calculados ajustando el modelo cinético a datos experimentales de fermentaciones con células en crecimiento tipo *repeated batch* y *fed-batch*.

Exp.		**33**	**35**
Ciclo	**Parámetro**	***Repeated batch***	***Fed-batch***
1	**k_P ($L \cdot g^{-1} \cdot h^{-1}$)**	$(3{,}02 \pm 0{,}11) \cdot 10^{-2}$	
	$Y_{X/P}$ ($g \cdot g^{-1}$)	$(3{,}41 \pm 0{,}15) \cdot 10^{-1}$	
	$Y_{S/P}$ ($g \cdot g^{-1}$)	$1{,}83 \pm 0{,}03$	
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	$(8{,}21 \pm 0{,}20) \cdot 10^{-1}$	
2	**k_P ($L \cdot g^{-1} \cdot h^{-1}$)**	$(1{,}51 \pm 0{,}04) \cdot 10^{-2}$	$(6{,}50 \pm 0{,}19) \cdot 10^{-3}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	$(2{,}65 \pm 0{,}16) \cdot 10^{-1}$	$(2{,}88 \pm 0{,}25) \cdot 10^{-1}$
	$Y_{S/P}$ ($g \cdot g^{-1}$)	$2{,}14 \pm 0{,}04$	$2{,}94 \pm 0{,}08$
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	$(9{,}46 \pm 0{,}22) \cdot 10^{-1}$	$(9{,}39 \pm 0{,}03) \cdot 10^{-1}$
3	**k_P ($L \cdot g^{-1} \cdot h^{-1}$)**	$(1{,}38 \pm 0{,}07) \cdot 10^{-2}$	$(2{,}33 \pm 0{,}01) \cdot 10^{-3}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	$(3{,}89 \pm 0{,}42) \cdot 10^{-1}$	0,00
	$Y_{S/P}$ ($g \cdot g^{-1}$)	$1{,}92 \pm 0{,}08$	$2{,}77 \pm 0{,}12$
	$Y_{SP/P}$ ($g \cdot g^{-1}$)	$(8{,}64 \pm 0{,}54) \cdot 10^{-1}$	$(4{,}45 \pm 0{,}05) \cdot 10^{-1}$
Ciclo	**Parámetro**	***Repeated batch***	***Fed-batch***
1	**F**	4715	
	RMSE	0,59	
	SSR	32,1	
	VE (%)	96,4	
2	**F**	8723	21562
	RMSE	0,24	0,25
	SSR	2,15	3,88
	VE (%)	99,7	98,8
3	**F**	1129	18932
	RMSE	0,80	0,32
	SSR	33,4	4,89
	VE (%)	96,0	96,1

Tabla 4.25. Valores de parámetros estadísticos y cinéticos calculados ajustando el modelo cinético a datos experimentales de fermentaciones con células en *resting* tipo *repeated batch* y *fed-batch*.

Exp.		**34**	**36**
Ciclo	**Parámetro**	***Repeated batch***	***Fed-batch***
1	**k_P ($L \cdot g^{-1} \cdot h^{-1}$)**	$(1,84 \pm 0,03) \cdot 10^{-2}$	
	$Y_{X/P}$ ($g \cdot g^{-1}$)	-	
	$Y_{S/P}$ ($g \cdot g^{-1}$)	$1,80 \pm 0,03$	
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	$(5,30 \pm 0,16) \cdot 10^{-1}$	
2	**k_P ($L \cdot g^{-1} \cdot h^{-1}$)**	$(5,36 \pm 0,14) \cdot 10^{-3}$	$(1,10 \pm 0,02) \cdot 10^{-2}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	-	-
	$Y_{S/P}$ ($g \cdot g^{-1}$)	$2,08 \pm 0,06$	$1,74 \pm 0,04$
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	$(9,17 \pm 2,49) \cdot 10^{-2}$	$(1,64 \pm 0,02) \cdot 10^{-1}$
3	**k_P ($L \cdot g^{-1} \cdot h^{-1}$)**	$(9,11 \pm 0,57) \cdot 10^{-4}$	$(4,43 \pm 0,10) \cdot 10^{-3}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	-	-
	$Y_{S/P}$ ($g \cdot g^{-1}$)	$3,80 \pm 0,25$	$(9,99 \pm 0,29) \cdot 10^{-1}$
	$Y_{SP/P}$ ($g \cdot g^{-1}$)	$(6,39 \pm 0,74) \cdot 10^{-1}$	$(1,24 \pm 0,21) \cdot 10^{-1}$
Ciclo	**Parámetro**	***Repeated batch***	***Fed-batch***
1	**F**	3512	
	RMSE	0,61	
	SSR	57,1	
	VE (%)	96,8	
2	**F**	5090	19278
	RMSE	0,49	0,28
	SSR	20,4	4,54
	VE (%)	94,8	98,5
3	**F**	25099	20931
	RMSE	0,27	0,36
	SSR	6,09	7,15
	VE (%)	90,7	96,5

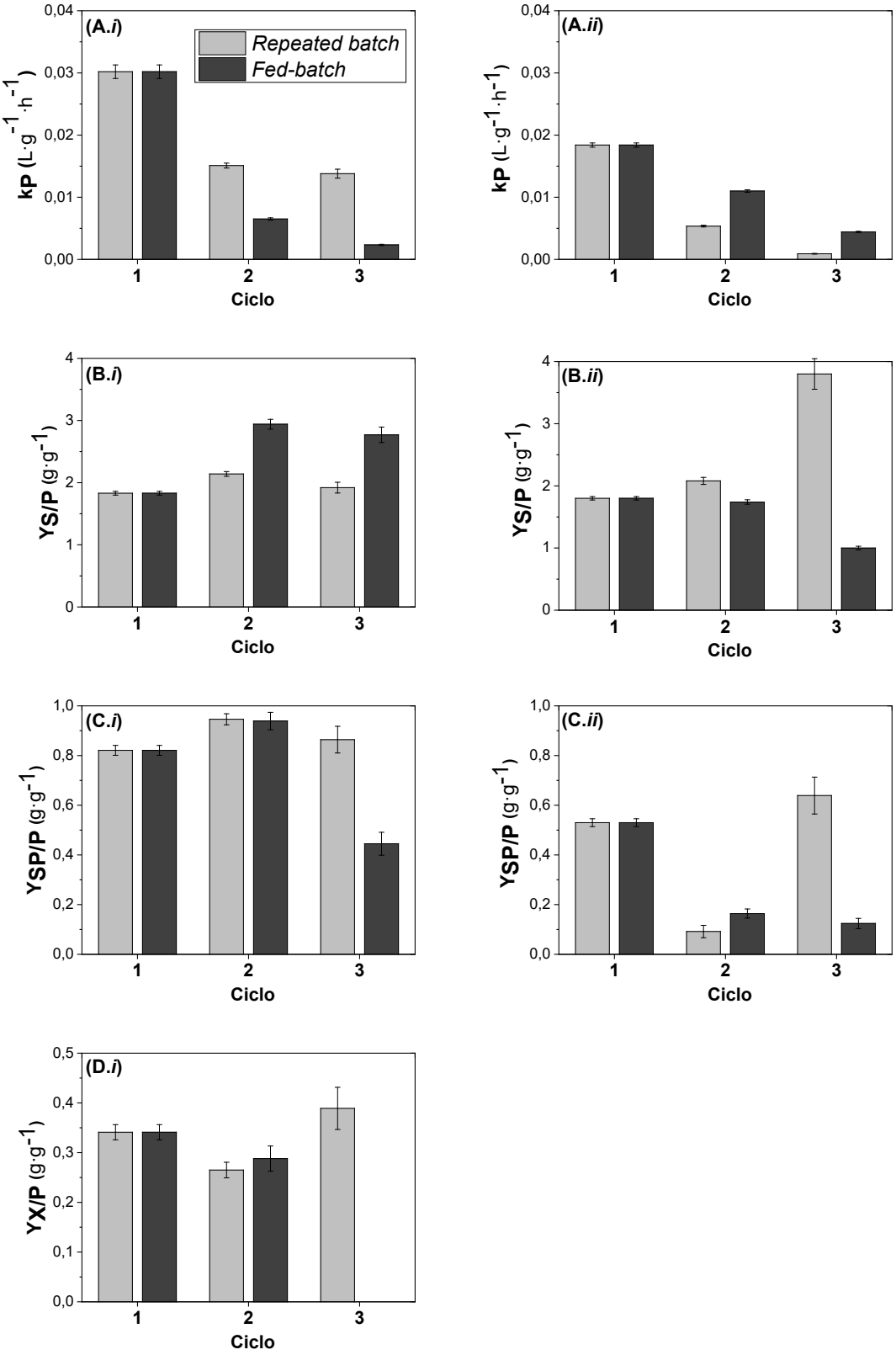

Figura 4.29. Influencia en los parámetros cinéticos de la reutilización de (i) células en crecimiento y (ii) células en *resting* en *repeated batch* y *fed-batch*. Exp. 33, 34, 35, 36.

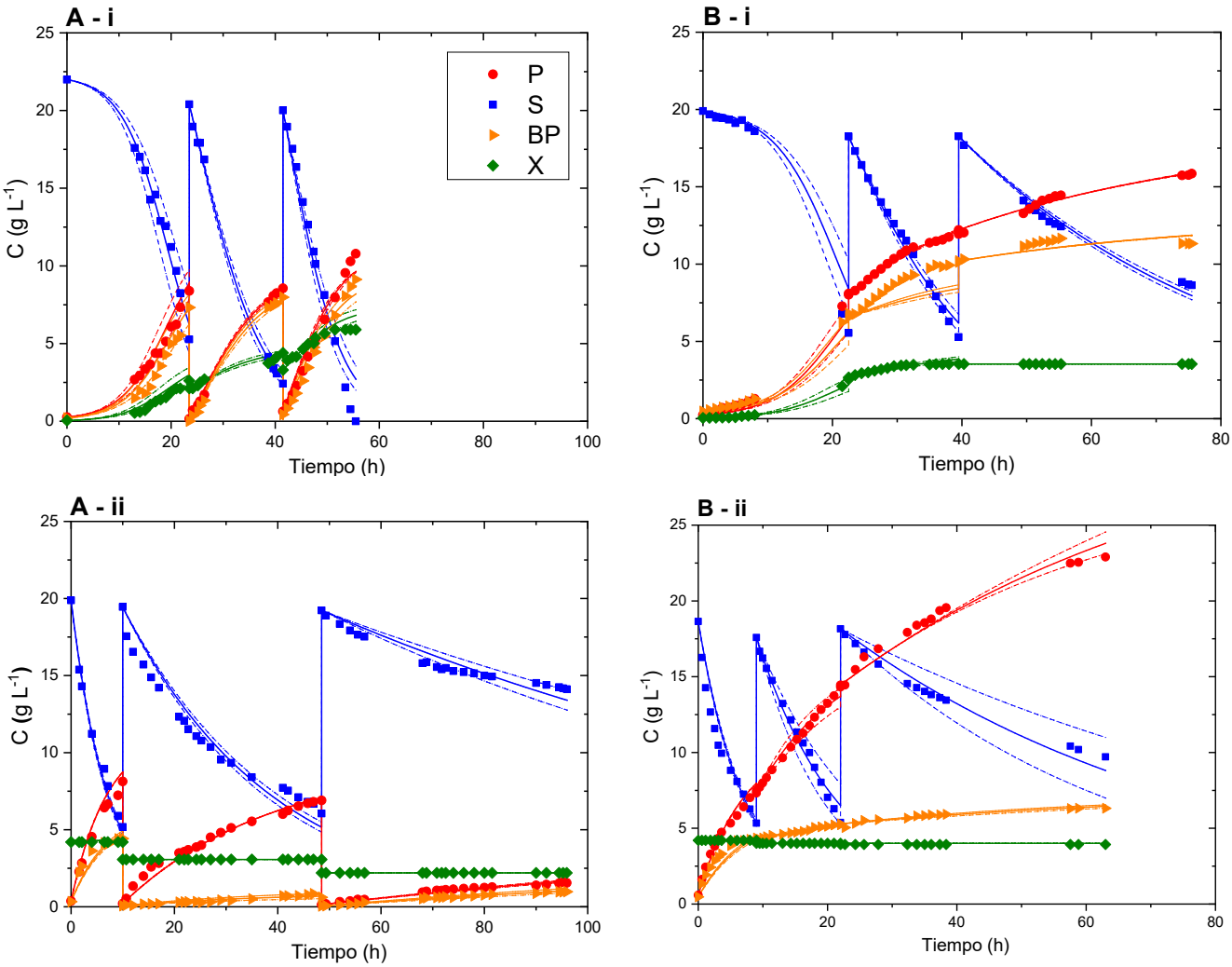

Figura 4.30. Evolución de las concentraciones de sustrato, producto, subproducto y biomasa operaciones tipo *repeated batch* (A) y *fed-batch* (B) con células en crecimiento (i) y células en *resting* (ii) – Exp.33, 34, 35, 36.

5. CONCLUSIONES

5 CONCLUSIONES

En esta sección se resumen las principales conclusiones obtenidas en esta Tesis Doctoral.

5.1 ADAPTACIÓN A LA FUENTE DE CARBONO Y PREPARACIÓN DEL INÓCULO.

- La adaptación de *A. succinogenes* a la fuente de carbono y la adición de una etapa de preinóculo aumentan la velocidad de crecimiento y la concentración final de biomasa en el inóculo.
- La adaptación a la fuente de carbono incrementa radicalmente el rendimiento y productividad de ácido succínico en reactor.
- La adición de una etapa de preinóculo mejora el proceso de fermentación.

5.2 ESTUDIO DE LA INFLUENCIA DE LAS VARIABLES Y CONDICIONES DE OPERACIÓN

- El proceso está influido por la concentración inicial de biomasa, siendo una concentración intermedia de inóculo (0,075 g L^{-1}) la que proporciona rendimientos y productividades más elevados.
- El intervalo de caudales de CO_2 generalmente empleado en la bibliografía sobre este proceso no presenta influencia en el proceso productivo por operar en exceso del gas.
- La agitación presenta influencia en el proceso, observándose un óptimo de rendimiento y productividad a 250 rpm y un posible estrés hidrodinámico por encima de ese valor.
- La concentración de extracto de levadura afecta de forma notoria tanto a la productividad (aumenta al aumentar la concentración empleada de este nutriente), como a la selectividad del proceso (disminuye al aumentar la concentración del nutriente). Sin embargo, los mayores rendimientos se alcanzan con 7,5 g L^{-1}.
- La concentración de la fuente de carbono (glucosa) influye en los parámetros de fermentación: a menores concentraciones se consiguen elevados rendimientos y productividades; sin embargo, la selectividad es mejor a concentraciones elevadas de azúcar.

5.3 ESTUDIO DE LA PRODUCCIÓN DE ÁCIDO SUCCÍNICO CON *RESTING CELLS*

- La adaptación de las células a la fuente de carbono es imprescindible para la viabilidad del proceso con células en estado de *resting cells*. La realización de una etapa de preinóculo mejora sustancialmente el rendimiento del proceso.
- Las células libres o en suspensión en la fase final de crecimiento exponencial son las más adecuadas para llevar a cabo el bioproceso con las células en estado de *resting cells.*
- Las células inmovilizadas en el *biofilm* tienen una actividad metabólica más elevada, por lo que si se realizara una operación con una mayor concentración de *resting cells* inmovilizadas probablemente los rendimientos de reacción serían mucho más elevados.
- El empleo de un medio más económico (MPEL) mejora el rendimiento y la productividad, pero empeora ligeramente la selectividad respecto del empleo del medio ampliamente utilizado en la literatura (TSB).

5.4 ESTUDIO DE FORMAS DE OPERACIÓN

- Las células en estado de crecimiento utilizadas en operación tipo *repeated batch* proporcionan los mejores resultados comparados con el empleo de operación tipo *batch* y *fed-batch*.
- Las células en estado de *resting cells* utilizadas en operación tipo *fed-batch* proporcionan los mejores resultados comparados con el empleo de operación tipo *batch* y *repeated-batch.*

5.5 USO DE HIDROLIZADOS COMO FUENTE DE CARBONO Y DE NITRÓGENO

- El uso de hidrolizados de residuos de patata como fuente de carbono (rico en glucosa) proporciona mayores rendimientos y productividades respecto al uso de glucosa pura, aunque se observa una cierta disminución en la productividad.

- El empleo de hidrolizado de bagazo de cerveza como fuente de carbono (rico en xilosa) proporciona mayores rendimientos y selectividades que el empleo de xilosa pura, pero una productividad ligeramente inferior.
- El factor de dilución del hidrolizado de levadura de cerveza gastada que se debe utilizar es de 6.
- El empleo de hidrolizado diluido de levadura de cerveza gastada como fuente de nitrógeno (y xilosa pura como fuente de carbono) proporciona mayores rendimientos y selectividades que con extracto de levadura comercial, aunque a velocidad de producción considerablemente inferior.
- El empleo simultáneo de hidrolizado de bagazo de cerveza (como fuente de carbono) e hidrolizado diluido de levadura de cerveza gastada (como fuente de nitrógeno), proporciona elevados rendimientos y selectividades, pero bajas velocidades de producción.
- El empleo como fuente de carbono de hidrolizado de residuos de patata en operación *repeated batch*, proporcionó los rendimientos y productividades máximos en la tercera etapa del proceso.
- El empleo simultáneo de hidrolizado de residuos de patata e hidrolizado diluido de levadura de cerveza gastada en operación *repeated-batch* proporciona un aumento del rendimiento en todas las etapas (comparado con el uso de extracto de levadura comercial como fuente de nitrógeno), aunque la velocidad de producción en la tercera etapa se ralentiza.
- La selectividad se ve claramente afectada, reduciendo su valor a lo largo de los ciclos en todos los experimentos independientemente de la fuente de nitrógeno empleada cuando se emplea hidrolizado de residuos de patata como fuente de carbono en operación *repeated-batch.*

5.6 INFLUENCIA DEL APORTE DE CO_2 EN EL PROCESO DE PRODUCCIÓN DE ÁCIDO SUCCÍNICO

- El estudio de la influencia del dióxido de carbono disuelto se debe llevar a cabo considerando todas las especies presentes en los equilibrios ácido-base del sistema: CO_2, HCO_3^- y CO_3^{2-} .

- Al valor de pH del proceso de producción (6,8), la distribución de las especies en el equilibrio es de un 25 % de CO_2 y de un 75 % de HCO_3^-
- La velocidad de transferencia de CO_2 en el sistema no es el fenómeno limitante de la velocidad global del proceso y no es necesario considerarlo en el modelo cinético.

5.7 MODELIZACIÓN CINÉTICA

- El empleo de la estrategia de *lumping* para los subproductos (ácidos acético y fórmico) proporciona muy buenos resultados en el ajuste de los modelos a los datos experimentales.
- El modelo cinético propuesto en este trabajo es capaz de ajustar todos los datos experimentales con gran fiabilidad estadística obtenidos en el proceso de producción de ácido succínico, considerando las diversas variables, medios de cultivo y formas de operación utilizadas con células en estado de crecimiento. Este modelo queda definido por las siguientes ecuaciones:

$$R_S = \frac{dC_S}{dt} = -Y_{S/X} \cdot \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_{Xm}}\right) - Y_{S/P1} \cdot k_{P1} \cdot C_S \cdot C_X - Y_{S/P2} \cdot k_{P2} \cdot C_S \cdot C_X$$

$$R_P = \frac{dC_P}{dt} = k_{P1} \cdot C_S \cdot C_X$$

$$R_X = \frac{dC_X}{dt} = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_{Xm}}\right)$$

$$R_{SP} = \frac{dC_{SP}}{dt} = Y_{S/SP} \cdot k_{P1} \cdot C_S \cdot C_X + k_{P2} \cdot C_S \cdot C_X$$

- El modelo cinético propuesto para fermentaciones con células en estado de *resting cells* es capaz de realizar un ajuste de gran bondad estadística a los datos experimentales obtenidos en este trabajo, independientemente de las diferentes variables y formas de operación consideradas. Las ecuaciones de velocidades de consumo de los sustratos y formación de los productos según el modelo desarrollado son las siguientes:

$$R_S = -Y_{S/P} \cdot k_p \cdot C_S \cdot C_X$$

$$R_P = k_p \cdot C_S \cdot C_X$$

$$R_{SP} = Y_{SP/P} \cdot k_p \cdot C_S \cdot C_X$$

6. ABREVIATURAS Y NOMENCLATURA

6 ABREVIATURAS Y NOMENCLATURA

6.1 ABREVIATURAS

ARAB: Arabinosa

ATP: *Adenosine TriPhosphate* (trifosfato de adenosina)

BHI: *Brain Heart Infusion* (infusion de cerebro y corazón)

BTX: Benzeno, Tolueno, Xileno

C: Carbono

CAGR: Compound Annual Growth Rate (tasa compuesta de crecimiento anual)

CAS: Chemical Abstracts Service (Servicio de resúmenes químicos)

CMNUCC: Convención Marco de las Naciones Unidas sobre el Cambio Climático

FAD: *Flavin Adenine Dinucleotide* (dinucleótido de flavina y adenine)

FAO: *Food and Agriculture Organization* (Organización para la agricultura y la alimentación)

FC: Fuente de Carbono

FD: Factor de Dilución

FDA: *Food and Drug Administration* (Administración de alimentos y medicamentos)

FN: Fuente de Nitrógeno

FRUCT: Fructosa

g: gas

GALACT: Galactosa

GEI: Gases de Efecto Invernadero

GLUC: Glucosa

GTIII: Grupo de Trabajo III

HLCG: Hidrolizado de Levadura de Cerveza Gastada

HMF: HidroxiMetilFurfural

HPLC: *High Performance Liquid Chromatography* (cromatografía líquida de alta resolución)

HRP: Hidrolizado de Residuos de Patata

IPCC: *Intergovernmental Panel on Climate Change* (Grupo intergubernamental de expertos sobre el cambio climático)

l: líquido

MALT: Maltosa

MAN: Manosa

MPR: Medio de Producción con células en *Resting*

NAD: *Nicotinamide Adenine Dinucleotide* (nicotinamida adenina dinucleótida)

NADH: *Nicotinamide adenine dinucleotide* reduced (nicotinamida adenina dinucleótida reducida)

NADPH: *Nicotinamide Adenine Dinucleotide Phosphate* reduced (nicotinamida adenina dinucleótido fosfato reducida)

NREL: *National Renewable Energy Laboratory* (Laboratorio nacional de energías renovables)

ODS: Objetivos de Desarrollo Sostenible

P: *Phosphate* (fosfato)

PEPC: *PhosphoEnolPyruvate Carboxylase* (fosfoenolpiruvato carboxilasa)

PEPCK: *PhosphoEnolPyruvate CarboxyKinase (*fosfoenolpiruvato carboxiquinasa*)*

PEP: *PhosphoEnolPyruvate (*fosfoenolpiruvato)

PET: Polietileno

PYC: *PYruvate Carboxylase (*piruvato carboxilasa)

SAC: Sacarosa

TCA: *TriCarboxylic Acid (*ácido tricarboxílico)

TSB: *Tryptic Soy Broth (*medio de digerido de soja y caseína*)*

UE: Unión Europea

USD: *United State Dollar (*dólar estadounidense)

US DOE: *United States Department Of Energy (*Departamento de energía de los estados unidos)

XIL: Xilosa

6.2 NOMENCLATURA

Abs: Absorbancia

Cj: Concentración del compuesto "j" (g L^{-1})

F: F de Fisher

k_P: constante cinética (L g^{-1} h^{-1})

K: número de parámetros, constante de equilibrio

N: número de datos experimentales

pj: presión parcial de "j" (atm)

Pj: productividad del compuesto "j" (g L^{-1} h^{-1})

ri: velocidad de la reacción "i" (g L^{-1} h^{-1})

Rj: velocidad de formación o consume del compuesto "j" (g L^{-1} h^{-1})

RMSE: *Root Mean Square Error*

Sj: selectividad del compuesto "j" (g g^{-1})

SSQ_l : suma de los residuos al cuadrado

SSQ_{meanl}: suma al cuadrado de las desviaciones entre los valores experimentales y los valores medios con respecto a los valores calculados

SSR: suma de residuos al cuadrado

VE: Variación Explicada

$y_{i,calc}$: valores calculados

Yi/j: rendimiento macroscópico de "i" sobre "j" (g g^{-1})

Yj: rendimiento del compuesto "j" (g g^{-1})

[CO_2]: concentración de CO_2 disuelto en el líquido (mol L^{-1})

[CO_3^{2-}]: concentración de ión carbonato (mol L^{-1})

[H^+]: concentración de protones (mol L^{-1})

[H_2CO_3] concentración de ácido carbónico (mol L^{-1})

[HCO_3^-] concentración de ión bicarbonato (mol L^{-1})

α: constante cinética, Ec. 4.15 ($L \cdot g^{-1} \cdot h^{-1}$)

β: constante cinética, Ec. 4.15 (L g^{-1})

μ: velocidad específica del crecimiento de biomasa (h^{-1})

6.3 SUBÍNDICES

a1: referido al equilibrio H_2CO_3 / HCO_3^-

a2: referido al equilibrio HCO_3^- / CO_3^{2-}

AA: referido a Acetic Acid (ácido acético)

EL: referido a Extracto de Levadura

G: referido a Glucosa

FA: referido Formic Acid (ácido fórmico)

H: referido a la constante de Henry

m: referido a un valor máximo

S: referido a Sustrato

SA: referido a Succinic Acid (ácido succínico)

SP: referido a SubProductos

S.cons: referido a Sustrato consumido

X: referido a biomasa

1,2…: referido al número de reacción

7. REFERENCIAS

7 REFERENCIAS

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7. ANEXO

8 ANEXO

8.1 PUBLICACIÓN 1

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Título: Modeling the Succinic Acid Bioprocess: A Review

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Resumen:

El ácido succínico ha atraído mucho interés como un químico de plataforma clave en el contexto de la bioeconomía, ya que se puede obtener en concentraciones elevadas a partir de biomasa a través de procesos de fermentación sostenibles, lo que supone una estrategia de producción valiosa para el futuro. Después de varios años de desarrollo del proceso de producción de ácido succínico, se han reportado muchos estudios sobre la producción a escala de laboratorio o piloto. Los datos experimentales relevantes revelan fenómenos dinámicos físicos y químicos subyacentes. Para aprovechar esta vasta, pero dispersa, información cinética, se han propuesto en primer lugar una serie de modelos cinéticos matemáticos del tipo no estructurado no segregado. Estos modelos relativamente simples presentan aspectos críticos de interés para el diseño, control, optimización y operación del bioproceso. Esta revisión incluye una descripción detallada de los fenómenos involucrados en los bioprocesos y cómo estos se reflejan en los modelos más importantes y recientes basados en la cinética química macroscópica y metabólica, y en algunos casos incluso en el transporte de masa.

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Review

Modeling the Succinic Acid Bioprocess: A Review

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Abstract: Succinic acid has attracted much interest as a key platform chemical that can be obtained in high titers from biomass through sustainable fermentation processes, thus boosting the bioeconomy as a critical production strategy for the future. After several years of development of the production of succinic acid, many studies on lab or pilot scale production have been reported. The relevant experimental data reveal underlying physical and chemical dynamic phenomena. To take advantage of this vast, but disperse, kinetic information, a number of mathematical kinetic models of the unstructured non-segregated type have been proposed in the first place. These relatively simple models feature critical aspects of interest for the design, control, optimization and operation of this key bioprocess. This review includes a detailed description of the phenomena involved in the bioprocesses and how they reflect on the most important and recent models based on macroscopic and metabolic chemical kinetics, and in some cases even coupling mass transport.

Keywords: succinic acid; fermentation; kinetic model; mass transport; phenomenology; carbon dioxide

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1. Introduction

The world gross domestic product (GDP) has been almost quintupled in the last 60 years due to intense global growth and the ever-growing search for the welfare state [1]. In this global scenario, the use of fossil fuels has grown at unprecedented rates. In fact, fossil fuel subsidies remained at USD 5.9 trillion in 2020, which represents 6.8% percent of the global GDP of that year [2]. At the current consumption rates, it is expected that only 14% of the present oil reserves, 72% of coal reserves and 18% of gas reserves will remain by 2050 [3,4]. In addition to the scarcity of these resources, the pernicious impact on health and the environment of the regular utilization of fossil fuels must be taken into account. [5–8].

As a logical consequence of the aforementioned issues and owing to a scenario of growing social concern for health and the environment, there has been a remarkable boost in research efforts towards the implementation of integrated biorefineries (IB). Their objective is the efficient use of biomass as a raw material for the production of biofuels, energy and chemicals in a single integrated facility [9,10]. Among the different IB types, those using lignocellulosic biomass as feedstock can be highlighted. Amidst their main advantages are the transformation of a wide variety of low-cost raw materials including, e.g., straw, reed, pruning…as well as the ability to obtain highly demanded products in markets currently covered by petrochemicals, with the prospects of opening new markets by producing further chemicals by organic synthesis [9–11].

One of the main challenges to overcome in biorefineries is the use of a type of biomass with long-term availability. Considering that biomass is any organic resource derived from plants or animals, we can count on lignocellulosic biomass, algal biomass, biomass

from food waste and industry or even municipal solid waste [12]. Lignocellulosic biomass is the most abundant renewable resource in the biosphere [13] and since it does not interfere in any food chain, this raw material is especially attractive in biorefinery processes [14]. It is generated by the photosynthesis process: the combination of CO_2 and H_2O using ultraviolet radiation from sunlight as an energy source results in sugars as the primary products with the subsequent production of O_2 as a by-product [15,16]. Lignocellulosic material consists of plant tissues whose cells have a wall made up of a network of cellulose microfibrils that group into larger fibers linked by hemicellulose. The structural integrity of these microfibers is protected by the presence of lignin, which acts as a linker [17,18].

However, the recalcitrance of lignocellulosic biomass demands on severe physical, chemical, physicochemical and biological pretreatments to leave the final solids accessible and reactive, thus rendering them available for hydrolysis into C5 and C6 monosaccharides. In consequence, these processes face several challenges to achieve high efficiencies and conversion yields without compromising their sustainability and cost-effectiveness [19,20].

Succinic acid (SA) is a key platform chemical for the bioeconomy due to the reactivity of its two functional carboxylic groups, which allows for obtaining a wide range of end-products [20,21]. In fact, according to the United States Department of Energy (US DOE), this compound is one of the target molecules that could be produced from biorefinery carbohydrates and from which many other products of interest may arise [22,23]. In fact, SA is considered the most important C4 building block and is widely used in several industries [24–26]

Since it has been demonstrated that SA has a positive influence on human metabolism, without the risk of bioaccumulation, it has been used in the food industry as an acidulant, flavoring agent and sweetener [27]. Moreover, in the traditional chemical industry, this compound has been key to the synthesis of other notable products, such as polybutylene succinate and polybutylene succinate-terephthalate, polyester or polyols, as well as in the pharmaceutical industry and in the production of resins, coatings and pigments. In addition, as the paradigm shift towards a bioeconomy evolves and becomes more and more established, SA has an outstanding potential for the generation of many intermediate chemicals of industrial relevance, such as 1,4-butanediol, butyriol-butone, tetrahydrofuran, n-methyl-2-pyrrolidone, 2- pyrrolidone, succinimide or maleic acid / maleic anhydride. Furthermore, succinate and its derivatives could be used for the manufacture of biodegradable polymers as polyamides and polyesters [19,21,28–30]. Figure 1 illustrates a diagram compiling the main products that can be obtained from SA.

Traditionally, the production of SA has been carried out through chemical technologies such as the oxidation of paraffins or the catalytic hydrogenation of maleic acid or maleic anhydride [21,27,31,32].

Microbial production of SA could become competitive due to several advantages, such as a high yield of SA from the conversion of carbon content or a significant reduction in greenhouse gas emissions and non-renewable energy consumption. Furthermore, a large amount of CO_2 is fixed during the process, which adds to the potential of this process as a mitigating strategy in the production of a commodity chemical [21,33].

Currently, the biotechnological production of SA prevails in the market compared to other routes. In 2011, the bioproduction of this compound accounted for less than 5% of the total production; however, only six years later, this share increased to practically 49% [34]. This increasing trend is expected to continue, predictably reaching a market volume of SA biotechnological production worth USD 2.22 billion by 2026, in contrast with the USD 170 million value in 2020 [35]. In realization of the expanding market for this product and the advantages offered by the biological production of SA, numerous companies have invested in the construction of industrial facilities for the production through this route. The production of this compound at an industrial level is especially advanced in Europe and North America, where its technological readiness level (TRL) is eight, which in turn means that there are complete and certified systems through tests and demonstrations

[21]. In 2015, succinic acid produced by bioprocesses had a market price of USD 2.86 per kg, while if it was obtained from fossil sources the price was around to USD 2.5 per kg [34]; however, as greenhouse gas emissions more than double during petrochemical production and the raw material costs are also higher (and their future availability uncertain), researchers and companies are increasingly turning to sustainable biological processes [21]. Currently, it has been possible to develop some economically competitive succinic acid production processes, with the acid produced having a market price between 2 and 2.5 USD kg^{-1} [36].

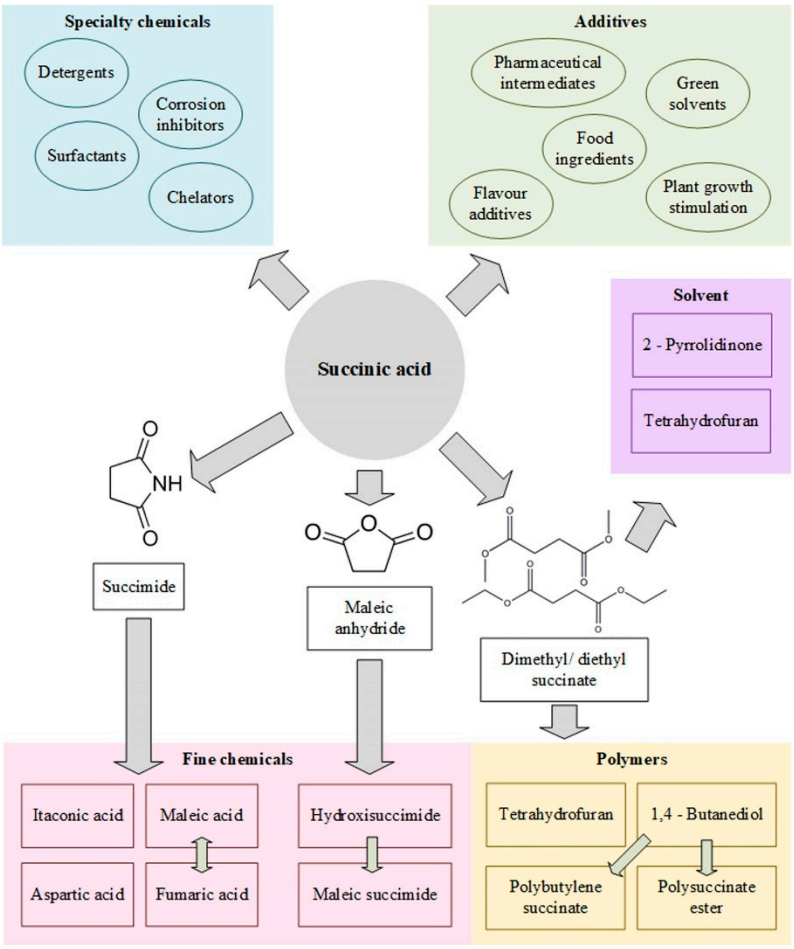

Figure 1. Array of intermediate and end products stemming from succinic acid.

1.1. Succinic Acid Bioproduction

According to the literature, the production of succinic acid is usually carried out between 33 and 39 °C at a controlled pH between 6.5 and 7. CO_2 is usually supplied to the system by insufflation in a gaseous state or by incorporating carbonates into the system, with $MgCO_3$ leading to the best production yields [19,37–44]. Most biological processes depend usually on the reaction or residence time (depending on the use of batch, fed-batch or continuous operating bioreactors); therefore, researchers have experimented with different configurations and types of reactors, managing to increase yields considerably with repeated batch and fed batch operating bioreactors [45–47]. In addition, continuous operation with a biofilm formation, in some cases, has resulted in highly improved productivities [48–51].

Almost all microbial, plant, and animal cells can generate SA; however, throughout the years it has been observed that the most suitable organisms for the production of this compound are fungi and bacteria [27]. Some fungi such as *Aspergillus niger*, *Penicillium viniferum*, *Yarrowia lipolytica*, and the yeast, *Saccharomyces cerevisiae*, generate SA as a by-

product of their metabolism in aerobic and / or anaerobic conditions [21,52]. The most investigated bacterial strains are *Actinobacillus succinogenes*, *Anaerobiospirillum succiniproducens*, recombinant *Escherichia coli*, *Corynebacterium glutamicum* and *Mannheimia succiniciproducens*, [29,52–55]. Being the isolates from the rumen (*A. succinogenes* and *M. succiniciproducens*), they are the ones that could obtain the most promising results, since they naturally generate C4 dicarboxylic acids during the pregastric digestion of polysaccharides. [25,29,52,56].

The latter microorganisms produce SA via the so-called tricarboxylic acid cycle (TCA), a cycle depicted in Figure 2. After sugars have been transformed into glyceraldehyde, bacteria convert phosphoenolpyruvate (PEP) (the last product of glycolysis) into oxalacetic acid, requiring CO_2 to activate this metabolic pathway. Oxalacetate is subsequently reduced in a series of steps until SA is produced [29,36]. From the reaction pathway showing in TCA, theoretically, to obtain 1 mol of SA, 2 moles of reduced nicotinamide adenine dinucleotide (NADH) and 1 mol of CO_2 would be necessary. As for sugars, 0.5 mol of glucose, 0.6 mol of xylose or 1 mol of glycerol would be necessary to generate another mol of succinate [26].

It is very important to highlight the different physiological features of SA-producing bacteria. *A. succiniproducens*, *A. succinogenes* and *M. succiniproducens* naturally produce SA as the main product of fermentation in the presence of CO_2 via the PEP carboxinase pathway [26,28]. It has been found that in the metabolism of *A. succiniproducens*, PEP carboxykinase (PEPCK) is the main CO_2-fixing enzyme for the generation of oxaloacetate. Furthermore, the conversion of pyruvate to acetyl-CoA is dependent on pyruvate-ferredoxin oxidoreductase; therefore, the control of pH and CO_2 concentration are critical in fermentations with *A. succiniproducens*, since PEPCK and pyruvate-ferredoxin oxidoreductase are strongly dependent on these parameters. Their optimization would lead to an increase in the productivity of succinic acid and a reduction in the generation of by-products (lactic acid, acetic acid and ethanol) [28,54]. *A. succinogenes* and *M. succiniproducens* have many aspects of their metabolism in common. In both cases, the main fermentation products are succinate, acetate and formate; however, the first of them can also generate ethanol, while the second would produce lactic acid. In its metabolism, oxalacetic acid is formed thanks to the action of PEPCK and is subsequently reduced to succinate by the C4 pathway. Although it is probably not the main enzyme in this process, it has been suggested that *M. succiniproducens* can also use PEP carboxylase (PEPC) for the carboxylation of oxalacetic acid [26]; however, PEPC is not encoded in the *A. succinogenes* genome. Succinic production does not take place simply due to part of the PEP branching to the C4 pathway. This must be taken into account since the increase in CO_2 concentration decreases the C4 decarboxylation flux, increases the pyruvate carboxylation flux and barely affects the PEPCK [26,28,54]. Although *E. coli* is capable of producing succinic acid through the reducing branch of tricarboxylic acid (TCA), it is not the main product of its fermentation naturally, which is why metabolic engineering has been used to increase production [26,29]. PEP, PEPC, and pyruvate-carboxylating enzymes have been overproduced to direct metabolic flux to the TCA-reducing branch. *A. succinogenes* PEPCK, pyruvate carboxylase (PYC) from *Lactococcus lactis* or *Rhizobium etli* have also been overproduced [26]. The *E. coli* NZN11 strain is capable of excreting pyruvate by increasing its carboxylation; however, it is not capable of fermentative growth and when malic enzyme is overproduced, the succinic production is greatly slowed down. [26,54]. Thanks to experiments on the transition from the aerobic growth phase to anaerobic production carried out with *E. coli*, it was discovered that, during aerobic growth, a new pathway was activated that involved the derivation of glyoxylate, using less reducing power and complementing the reducing branch of TCA [26]. With the AFP111 and SBS550MG/pHL413 strains, the aim was to eliminate the fermentation by-products and guarantee the glyoxylate bypass flow; however, it must be taken into account that if an additional reductant could be used, the glyoxylate route would be less efficient than in the case of maximizing the flow of the reductant TCA branch. *C. glutamicum* in anoxic conditions, with carbonate and when growth is absent, is

capable of producing succinic, lactic and acetic acids. In this microorganism, oxalacetic acid is produced mainly thanks to the action of PEPC with less contributions from PEPCK and PYC, later this compound gives rise to succinic acid through the reductive branch of the TCA cycle, not being necessary the glyoxylate shunt [26,54].

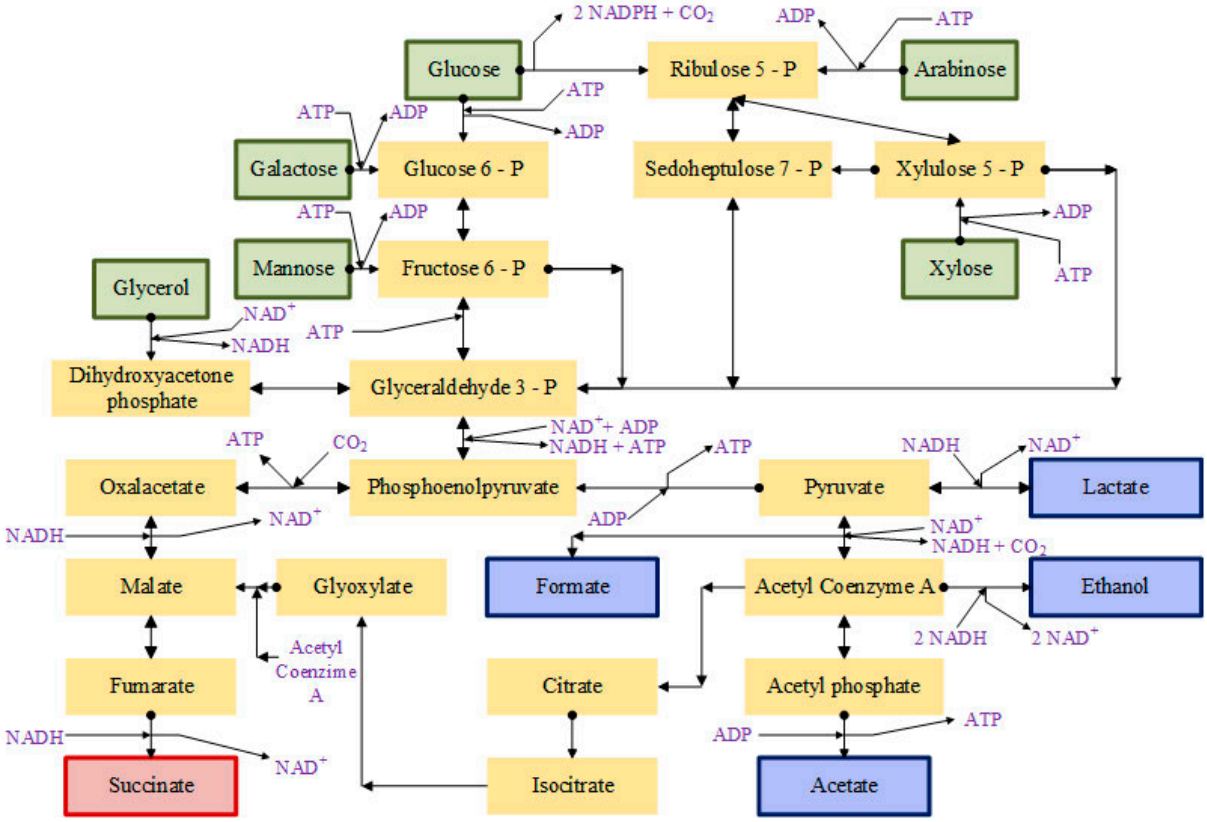

Figure 2. Metabolic pathways in succinic acid-producing microorganisms such as *A. succinogenes*, *A. succinoproducens*, *M. succiniproducens* and anaerobic succinate engineered *E. coli*.[26,28,29], Adenosine triphosphate (ATP), flavin adenine dinucleotide (FAD^+), nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), and reduced nicotinamide adenine dinucleotide phosphate (NADPH).

1.2. The Importance of Kinetic Modeling

In light of the relevance of SA in the context of biorefineries and how these will become a trend spurred by global policies, this review intends to provide an extensive overview of empirical models that describe its biotechnological production. The formulation of models capable of describing the evolution of the compounds involved in fermentation is of great importance in the industrialization of biotechnological processes. These models allow the simulating of the temporal behavior of the system, which, from an engineering point of view, is essential for the development and scale-up of the process. Taking into account that the performance and productivity of a process not only depend on the genetic constitution of cells, but also on the way in which fermentation is carried out, these models are especially useful for the choice of the reactor and its design. Furthermore, these models can also be implemented in the design a control system, and will definitely aid during scale-up and industrial process simulation and implementation through diverse techno-economic studies. In fact, a mathematical description of the process, typical in process systems engineering [57], allows its optimization through simulation, saving time, effort and resources for experimentation at several scales. As a consequence, differential equations are established for the rates at which biochemical reactions are taking place. This set of equations are usually called kinetic models, although they are also called dynamic models, due to the overlapping of concepts in terms of biological models [58–60].

Therefore, to conduct fermentation processes at an industrial level implies the consideration of different amounts of materials and energy inputs at each production level, which can affect the behavior of the microorganism; therefore, it is necessary to approach an isolated study of different individual phenomena and variables on a laboratory scale prior to their simultaneous consideration. In this way, proper coupling of the phenomena can then accurately describe the overall process [60,61], as shown in Figure 3, from the point of view and language of chemical reaction engineering.

The major dynamic phenomena to take into account are the following:

- Mass transfer among phases. Due to the fact that microbial systems for the production of succinic acid are heterogeneous (gas–liquid–solid), it is essential to study the transport of nutrients among phases in the system [29,36,41,42].
- Reaction kinetics in the system. The description of a bacterial reaction network is very complex, although the evolution of the concentrations can be described through a kinetic model [62].
- Stress suffered by the cells. Due to the hydrodynamic conditions and the presence of radical species (ROS) and other operational parameters, the physiology of the cells may undergo severe changes throughout the bioprocess, which may be deleterious to their performance [58,61–63].

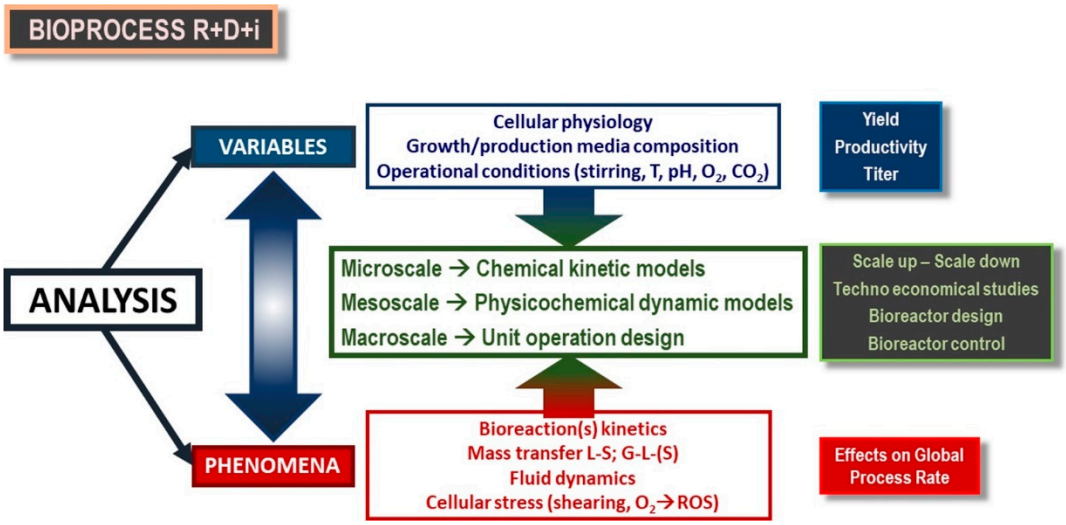

Figure 3. Dynamic phenomena coupling and modelling in a research, development and innovation (R+D+i) scheme for the implementation of a bioprocess.

2. Kinetic Models

Through microbial kinetics, the manifestations and reactions of microbial life can be studied, namely, growth, survival, death, adaptations, product formation, cell cycles and interactions with the environment; therefore, to determine the economic loss or gain of the processes, it is essential to establish models that represent their kinetic behavior. The formulation of the kinetic model consists of the approach of a simplified network of reactions that describes the lumped chemical transformations that take place as well as the kinetic equations that determine the rate at which each of the reactions involved in the network takes place. The proposed kinetic equations are differential equations that can derive from phenomenological hypotheses or empirical descriptions. From the reaction scheme and the reaction rates, the production rate of each compound is proposed, taking into account the reactions in which each one is involved and their stoichiometry. The complexity of the study of biochemical metabolism lies in the network of reactions that take place, since routes are distinguished both for the synthesis of complex molecules and the precursors of intermediates (anabolic route) and for the supply of the necessary energy in

the anabolic processes (catabolic routes). Such models can have different degrees of complexity and provide different extents insights into the underlying phenomena; thus, they can take the shape of correlations that range from purely empirical (black box) to fully mechanistic (white box) ones. In any case, they help establish relationships between the reaction rates and concentrations of the species inserted in material balances and allow the prediction of conversion degrees and yields [62].

Due to its simplicity, currently, the most widespread models are those that consider the microorganism as a single component (biomass), in other words, the so-called unstructured-non-segregated models; however, there are other more complex models. In metabolic models, although they are also normally unstructured-non-segregated, the metabolic pathways are described as a network of reactions using a simplified reaction scheme, with defined stoichiometric relationships. When the description of biomass is made considering it to be made up of several species, taking into account the intracellular components, it is a structured model (or cell model). The chemically structured models consider the biomass formed by several species and also a simplified metabolism (a network of reactions). The segregated models describe the microorganism considering the distribution of some property, that is, they do not consider an average microorganism but diverse microbial populations [58,64,65].

Numerous research efforts have focused on studying the kinetic evolutions of the compounds involved in the SA production process using different microorganisms. This has allowed conclusions to be drawn on general growth trends and inhibitory behaviors. From the performance of fermentations operating in batch, repeated batch and fed batch modes, it has been possible to observe how the SA-producing microorganisms show certain behaviors in the consumption of substrates and the generation of products and by-products [38,66,67].

Comparing the time evolution of SA together with the trend of biomass, it can be observed that, regardless of the microorganism or substrate used [67–72], the main product begins to generate before the biomass reaches the steady state. Moreover, the product concentration continues to grow during the stationary phase of the microorganism; therefore, it can be deduced that the production of SA is partially associated with the growth of biomass.

It is known that some compounds present in the hydrolysates of lignocellulosic raw materials, such as furfural, hydroxymethylfurfural or phenolic compounds, act as inhibitors; therefore, their concentrations in the culture broth are factors that must be taken into account when studying the kinetics of the process [19,73,74]. However, other factors that may affect the activity of the microorganisms, and, therefore, the kinetic models and their parameters, must also be considered. It is necessary to maintain adequate pH levels, whose optimal value is usually considered to be 6.8. Another variable to take into account is the nature of the compound used as a regulator. Under anaerobic conditions, it has been observed that the replacement of typical hydroxides (KOH, NaOH) by carbonates (K_2CO_3, Na_2CO_3, $MgCO_3$) leads to higher cell viability and, therefore, to a higher final concentration of SA [75,76]. Furthermore, $MgCO_3$ can also act as a source of CO_2, and the infusion of this gas can be totally or partially substituted, maintaining the yields or even exceeding them [76]. Osmolarity has also proven to influence the SA production process, but its effects can be considered negligible compared to those generated by organic acids in the medium such as SA, FA, AA and PA [38,39,70,77,78]. Inhibitions by substrates must also be considered, since it has been verified that both high concentrations of glucose [38,77,79] and xylose [39,80] generate this effect.

Non-Segregated, Unstructured Models

Non-segregated unstructured models are the most widely used in chemical engineering. Depending on the variables they consider, a wide variety of them with different degrees of complexity can be distinguished [60].

2.1.1. Biomass Growth Models

First, there are the models that consider biomass concentration as the only determining factor for its growth. The most representative equations are Malthus' Law for the exponential phase in steady state systems—Equation (1) and the logistic equation—Equation (2)—[81]:

$$\frac{d[X]}{dt} = \mu \times [X] \quad (1)$$

$$\frac{d[X]}{dt} = \mu \cdot [X] \times \left(1 - \frac{[X]}{[X]_m}\right) \quad (2)$$

where *[X]* is the biomass concentration, *t* is time, μ is the specific growth rate and $[X]_m$ is the maximum biomass concentration.

Another type of equation is presented in Equation (3), in which the growth of biomass is dependent on the concentration of biomass and the limiting substrate (*[S]*), the latter dependence being included in the specific growth rate:

$$\frac{d[X]}{dt} = \mu([S]) \times [X] \quad (3)$$

Blackman, M'Kendric and Pai and Tiesser proposed expressions for the calculation of the specific growth rate considering the influence of the limiting substrate: however, the Monod model—Equation (4)—is considered a fundamental equation in microbial kinetics and is also the most widely used for the description of biomass in the production of SA [39,78,82]. This empirical model is an analogy to the Michaelis Menten model for unisubstrate irreversible enzymatic reactions and is based on the enzymatic nature of the reactions that take place inside the microorganism [39,61]:

$$\mu = \frac{\mu_m \times [S]}{K_S + [S]} \quad (4)$$

In this equation, μ_m is the maximum specific growth rate and K_S is the saturation constant of the substrate.

Deriving from this model, numerous expressions have been developed to describe the growth of biomass considering the inhibition by substrate. The main expressions that have been used for the modelling of SA generation experiments are shown below—Equations (5) – (12)—which derive either by a combination of some of them [38,40,83] or by application in their original form [56,82,84]:

Mosser $$\mu = \frac{\mu_m \times [S]^n}{K_S + [S]^n} \quad (5)$$

Tessier $$\mu = \mu_m \times \left(1 - e^{[S]/K_S}\right) \quad (6)$$

Tessier (high [S]) $$\mu = \mu_m \times \left(e^{-[S]/K_{IS}} - e^{-[S]/K_S}\right) \quad (7)$$

Haldane–Andrews $$\mu = \frac{\mu_m \times [S]}{[S] + K_S + \left(\frac{[S]^2}{K_{IS}}\right)} \quad (8)$$

Andrews (high [S]) $$\mu = \frac{\mu_m}{\left(1 + \frac{K_S}{[S]}\right) \times \left(1 + \frac{[S]}{K_{IS}}\right)} \quad (9)$$

Aiba–Edward $$\mu = \frac{\mu_m \times [S]}{[S] + K_S} e^{-[S]/K_{IS}} \quad (10)$$

Luong $$\mu = \frac{\mu_m \times [S]}{[S] + K_S} \cdot \left(1 - \frac{[S]}{[S]_m}\right)^{\alpha} \quad (11)$$

Jerusalimsky $$\mu = \mu_m \left(\frac{1}{1 + [S]/K_{IS}}\right) \quad (12)$$

In these equations, n indicates the degree of inhibition (n = 1 in the Monod model), K_{IS} is the inhibition constant per substrate and α is a parameter relating μ and *[S]*.

Since the inhibition by product also occurs during fermentation, other models have been developed to take this phenomenon into account. When this is the case, the specific growth rate can be expressed by Equation (13):

$$\mu = \frac{\mu_i \times [S]}{K_S + [S]} \quad (13)$$

where μ_i is the maximum specific growth rate in the presence of an inhibitor. In studies of the kinetics of SA generation [41,56,79,85], the main expressions used are those corresponding to Equations (14) to (16):

Luong $$\mu_i = \mu_m \times \left(1 - \frac{[P]}{[P]_m}\right)^{\beta} \quad (14)$$

Aiba–Edward $$\mu_i = \mu_m \times e^{-[P]/K_{IP}} \quad (15)$$

Jerusalimsky $$\mu_i = \mu_m \times \left(\frac{1}{1 + [P]/K_{IP}}\right) \quad (16)$$

in which *[P]* is the product concentration, β is the reaction between μ and *[P]* and K_{IP} is the inhibition constant per product.

2.1.2. Substrate Consumption Models

Equation (17) shows the simplest expression for obtaining the rate of substrate consumption. It is based on the relationship between the coefficient of yield of substrate in biomass (Y_{SX}) and the specific growth rate, and is one of the most used in the kinetic study of the production of SA.

$$\frac{d[S]}{dt} = \mu \times Y_{SX} \quad (17)$$

However, several researchers have considered that the substrate is also consumed to maintain biomass in a viable state throughout the reaction. For this reason, they have introduced the so-called Pirt's maintenance coefficient m [38,40,83,86] in the substrate consumption equations. Furthermore, some of these authors have also included a term in their models that allows for describing the consumption of energy substrate for production [38,83,86], as shown in Equations (18) and (19):

$$\frac{d[S]}{dt} = \left(-\frac{1}{Y_{XS}} + \sum_I \frac{1}{Y_{PiS}}\right) \times \frac{d[X]}{dt} + \left(-\sum_I \frac{1}{Y_{PiS}} + m\right) \times [X] \quad (18)$$

$$\frac{d[S]}{dt} = \left(-\frac{1}{Y_{XS}}\right) \times \frac{d[X]}{dt} + \left(-\sum_i \frac{1}{Y_{PiS}}\right) \frac{dP_i}{dt} + (-m) \times [X] \quad (19)$$

2.1.3. Product Generation Models

Most kinetic studies on the biological production of SA predict the generation rate of this compound and of the by-products through the Luedeking–Piret expression—Equation (20)—as is logical in a production process partially affected by the growth of the biomass [38,83,86,87].

$$\frac{d[P_i]}{dt} = \alpha_i \times \frac{d[X]}{dt} + \beta_i \times [X] \quad (20)$$

where $[P_i]$ is the concentration of SA or by-product, α_i is the associated growth parameter and β_i is the non-associated growth parameter; however, in some cases simpler expressions have been used, such as the one used in Equation (21), where the generation of SA only depends on the specific growth rate [88]. In other instances, expressions of greater complexity have been regarded, such as the modified Gompertz model [89] as seen in Equation (22), where $[P_m]$ is the maximum metabolite concentration, R_m is the maximum rate of metabolite production, e is the Euler number and λ is the time of the latency phase.

$$\frac{d[P]}{dt} = \mu \times Y_{PX} \quad (21)$$

$$[P] = [P_m] \times exp\left(-exp\left[\frac{R_m \times e}{[P_m]}(\lambda - t) + 1\right]\right) \quad (22)$$

For experimental data obtained in continuous processes, relatively simple empirical expressions have been proposed to describe SA production [85]. On the other hand, Ferone et al. proposed Equation (23) [90], where the variation of the concentration of SA is represented as a function of the dilution rate (D). Under steady state conditions, D is equivalent to the specific growth rate.

$$\frac{d[P]}{dt} = \frac{D \times [P]}{[X]} \quad (23)$$

Table 1 presents a summarized compilation and classification of kinetic studies carried out on the bioproduction of SA in recent years, in which non-segregated unstructured models are proposed and fitted to experimental data.

Lin et al., Pateraki et al., Song et al., Vlysidis et al. and Li et al. [38,40,83,86,91] have developed some of the most complete models of this type, predicting the evolution of the concentration of biomass, substrate, SA and by-products in reactions catalyzed by *A. succinogenes*, *B. succiniproducens*, *M. succiniproducens* and *Y. lipolytica*. Table 2 shows a comparison of the parameters that describe the growth of biomass according to these authors. In all cases, a Monod model was used with inhibitions by a Haldane–Andrews type substrate and Luong type product, except in the case of the study carried out by Lin et al. [38], who have considered that an inhibition by substrate also follows a Luong model.

As can be observed, the value of μ_m—Equations (5), (8), (11) and (14)—is between 0.12 and 1.324 h^{-1} depending on the microorganism and environmental factors. Some of the lowest values of this activity parameter are found in fermentations by *A. succinogenes*, being the lowest when glycerol was used as the substrate [86], followed by the one corresponding to the use of a mixture of sugars rich in xylose [40]. The maximum growth rate of *A. succinogenes* was reached using glucose as the carbon source [38], more than four times higher than when using glycerol [86]. Among the fermentations carried out with glucose as the carbon source, considerably higher μ_m values were achieved in the case where the biocatalyst was *M. succiniciproducens* [83] instead of *A. succinogenes* [38]. In the cases in which glycerol was used as a substrate, this parameter tripled when *Y. lipolytica* [91] was selected instead of *A. succinogenes* [86].

Table 1. Non-segregated unstructured kinetic models.

Microorganism	Operation Mode	Carbon Source	Species Predicted by the Model				Ref.
			Biomass	Substrate	Product	By-products	
A. succinogenes 130Z, *E. coli* NZN111, AFP111, BL21	Batch	Glucose	Eq. (1), (2)				[78]
A. succinogenes DSM 22257	Batch	Glucose, mannose, xylose, arabinose	Eqs. (1), (4), (8)				[39]
E. coli ATCC 8739	Batch	Glycerol	Eqs. (1), (4) – (10)				[82]
B. succiniciproducens BPP7	Fed Batch	*Arundo Donax* hydrolysate	Eq, (1), (14)				[41]
A. succinogenes 130Z	Batch	Glucose	Combination of Eqs. (1), (12), (16)	Eq. (17)			[79]
A. succinogenes DSM 22257	Continuous	Glucose	Combinations of Eqs. (1), (4), (8), (11), (14) – (16)		Eq. (23)		[90]
A. succinogenes 130Z	Batch	Oil palm frond hydrolysate	Eq. (8)		Eq. (22)		[89]
A. succinogenes 130Z	Continuous	Glucose	Empirical		Empirical		[85]
A. succinogenes 130Z	Batch, Fed Batch	Raw carob pod extracts	Eq. (1), (4)	Eq. (17)	Eq. (20)		[87]
A. succinogenes ATCC 55618	Batch	Glucose	Combination of Eqs. (1), (11), (14)	Eq. (18)	Eq. (20)	Eq. (20)	[38]
A. succinogenes ATCC 55618	Batch	Glycerol	Combination of Eqs. (1), (8), (14)	Eq. (19)	Eq. (20)	Eq. (20)	[86]
A. succinogenes 130Z, *B. succiniciproducens* JF 4016	Batch	Xylose, galactose, glucose, mannose, arabinose.	Combination of Eqs. (1), (8), (14)	Eq. (18)	Eq. (20)	Eq. (20)	[40]
M. succiniciproducens MBEL55E	Batch	Glucose	Combination of Eqs. (1), (8), (14)	Eq. (19)	Eq. (20)	Eq. (20)	[83]
Y. lipolytica PGC0100	Batch	Glycerol	Combination of Eqs. (1), (8), (14)	Eq. (19)	Eq. (20)	Eq. (20)	[91]

Table 2. Parameters from non-segregated unstructured biomass growth kinetic models.

Microorganism	Carbon source	Eqs.	Parameters							Ref.
			μ_m h^{-1}	$[S]_m$ g L^{-1}	$[P]_m$ g L^{-1}	K_S g L^{-1}	K_{IS} g L^{-1}	α	β	
A. succinogenes ATCC 55618	Glucose	(1) (11) (14)	0.5	155	SA-104.2 ET-42.1 AA-44.2 FA-16.0 PA-74.1	2.03		0.603		[38]
A. succinogenes ATCC 55618	Glycerol	(1) (8) (14)	0.12		45.6	2.896	15.36		1.074	[86]
A. succinogenes 130Z	Xylose galactose glucose mannose arabinose	(1) (8) (14)	0.394		AA-38 FA-18 LA-60 SA-55	0.698	55.484		AA-2.300 FA-2.300 LA-/ SA-2.300	[40]
B. succiniciproducens JF 4016			0.932		AA-38 FA-22 LA-58 SA-55	1.556	15.173		AA-2.300 FA-2.299 LA-2.300 SA-2.299	
M. succiniciproducens MBEL55E	Glucose	(1) (8) (14)	1.324		17.23	1.123	88.35		1.301	[83]
Y. lipolytica PGC0100	Glycerol	(1) (8) (14)	0.38		AA-57.9 SA-243.4	0.818	223.5		AA-12.02 SA-12.30	[91]

The values of the limiting nutrient concentration at which the specific growth rate is half of the maximum K_S—Equations (5), (8), (11)—are between 0.698 g L^{-1} and 2.896 g L^{-1}. With *A. succinogenes*, Vlysidis et al. [86] achieved the highest value employing glycerol as the carbon source. The value estimated by Lin et al. [38] with the same microorganism turned out to be quite high as well (2.03 g L^{-1}) when glucose was used as a carbon source, but when opting for a carbon source rich in xylose, this value dropped drastically to 0.698 g L^{-1}, as observed by Pateraki et al. [40]. In fermentations with glucose, using *A. succinogenes*, the K_S was practically double [38] of that with *M succinoproducens* [83]. In the operations with glycerol, the estimates made from the data obtained from the production carried out by *A. succinogenes* [86] were considerably higher (2.896 g L^{-1}) than when *Y. lipolytica* was used (0.818 g L^{-1}) [91]. Among the selected fermentations, an evaluation of the reported values of K_{IS}—Equations (8) and (11)—allows to conclude which are the strongest inhibitions due to the substrate. When working with *A. succinogenes*, a substrate mixture rich in xylose (55.48 g L^{-1}) turns out to be much less inhibitory [40] than glycerol (15.36 g L^{-1}) [86]. It should be noted that, when glycerol is selected as the carbon source, inhibition falls when, instead of opting for this microorganism, *Y. lipolytica* yeast (223.5 g L^{-1}) is used [91].

According to the $[P]_m$ calculated by Lin et al. [38], the products that generated inhibition in the growth of *A. succinogenes* with glucose are SA, ethanol (ET), AA, FA and PA. Pateraki et al. [40], starting from a mixture of xylose and other sugars, observed inhibition by AA, FA, LA and SA. Vlysidis et al. [86] preferred to neglect the possible inhibitory effect of the by-products, including the assumption that only SA generates considerable inhibition in glycerol fermentation. Although Lin et al. [38] considered the inhibitory effect of various compounds using glucose as the carbon source, Song et al. [83] only included SA in their estimates in a process carried out with the same substrate but with *M. succiniproducens* as the biocatalyst. In the operations with glycerol, it seems that there are fewer species with inhibitory effects, with only SA being considered in both the studies by Vlysidis et al. [86] as in those of Li et al. [91], although the latter authors also considered the influence of FA.

Table 3 shows the parameters obtained by Lin et al., Pateraki et al., Vlysidis et al., Song et al. and Li et al. [38,40,83,86,91] in the study of the evolution of the concentrations of substrates and products in the fermentations. Lin et al. and Pateraki et al. [38,40] have proposed models in which the parameters of the kinetic models of substrate concentration are grouped into two constants. The first of them is δ (Equation 18), which is associated with growth, thus encompassing the yields of biomass and product in a substrate). The other parameter is γ (Equation 18), which is not associated with the growth of biomass but includes the yield of product in a substrate and the Pirt's maintenance coefficient. On the other hand, Vlysidis et al., Song et al. and Li et al. [83,86,91] have considered that the yield of biomass in a substrate would be associated with the growth of biomass, whilst the yield of product in a substrate would be associated with the production of acid and the Pirt's maintenance coefficient is multiplied by the biomass concentration (Equation 19). Both δ (4.35-7.575 g g^{-1}) and γ (0.034-0.308 g g^{-1} h^{-1}) are observed to reach the highest values in reactions catalyzed by *A. succinogenes*, especially when using a mixture of sugars rich in xylose [40]. Vlysidis et al., Song et al., Li et al. and [83,86,91] estimated that the evolution of the substrate could be studied considering exclusively the following parameters: the yield of this carbon source in biomass, the yield to SA in the substrate and the Pirt's maintenance coefficient, the latter of which acquires a value practically of zero.

Table 3. Parameters from non-segregated unstructured kinetic models considering substrate consumption and product generation in SA production.

Microorganism	Carbon Source	Substrate						Product and By-Products			Ref.
		Eqs.	Parameters					Eqs.	Parameters		
			Y_{XS}	Y_{PiS}	δ g g^{-1}	γ g g^{-1} h^{-1}	m_s h^{-1}		α_i g g^{-1}	β_i g g^{-1} h^{-1}	
A. succinogenes ATCC 55618	Glucose	(18)			4.35	0.308		(20)	AA-1.430 FA-0.881 PA-0.187 SA-3.600	AA-0.045 FA-0.013 PA-0.049 SA-0.299	[38]
A. succinogenes ATCC 55618	Glycerol	(19)	0.130	2.790			0.001	(20)	AA-0.753 FA-0.428 SA-9.864	AA-0.001 FA-0.002 SA-0.001	[86]
A. succinogenes 130Z	Xylose galactose glucose mannose arabinose	(18)			7.575	0.051		(20)	AA-2.258 FA-1.882 LA-/ SA-3.858	AA-2.136 FA-1.501 LA-0.419 SA-4.080	[40]
B. succiniciproducens JF 4016					6.685	0.034			AA-0.016 FA-0.006 LA-/ SA-0.040	AA-0.001 FA-0.001 LA-0.001 SA-0.028	
M. succiniciproducens MBEL55E	Glucose	(19)	0.765	AA-0.999 FA-1.532 LA-0.999 SA-1.310			0.061	(20)	AA-0.626 FA-0.665 LA-/ SA-1.619	AA-0.124 FA-0.105 LA-0.210 SA-0.355	[83]
Y. lipolytica PGC0100	Glycerol	(19)	0.581	AA-1.864 SA-1.712			0.055	(20)	AA-0.208 SA-0.386	AA-0.010 SA-0.013	[91]

It should be noted that, despite starting from glycerol in both cases, Vlysidis et al. [86] only considered the effect of SA carrying out the fermentation with *A. succinogenes*, while Li et al. [91] have also had to consider the inhibition generated by AA when using *Y. lipolytica* as a biocatalyst.

All the authors of these works consider that AA and SA produce an inhibitory effect on SA production—Equation (20)—[38,40,83,86,91]. Regarding the parameters associated with the growth of the microorganism, it can be observed that the effect of SA is the one

with the highest values. For all microorganisms and substrates, the coefficient not associated with growth presents higher values for SA, except in the case of fermentation with *A. succinogenes* starting from glycerol [86], where the acid that generates the greatest impact in productivity is PA.

3. Mass Transfer Phenomena

In the previous section, we have compiled key information on the work of researchers that studied the evolution over time of the species involved in fermentation, proposing kinetic models and estimating parameters that allow for optimizing the system. However, to predict the distribution of the chemical species in the different phases in contact involved in a fermentation process, it is essential to conduct a preliminary analysis on the mass transfer among these phases. It is the joint knowledge and coupling of bio/chemical kinetics, heat and mass balances and transport phenomena that allows for carrying out an adequate design of the reactor and of the control system as well as the realization of a change and techno-economic studies, as explained above.

Microbial processes usually take place in triphasic systems (gas–liquid–solid), where the continuous phase is liquid, while the gas phase and the solid microorganisms can be considered as discontinuous phases in suspension or travelling through the liquid phase, usually aqueous. Nutrients and metabolites present a different resistance to transport depending on their molecular volume and the fluid dynamics of the phase or interface they are in. Nutrients' availability at the cellular level is key to achieving high yields. In addition, the gas–liquid transport of CO_2 in these systems acquires special relevance as CO_2 activates the SA generation route.

Until now, the studies carried out on the mass transfer of CO_2 are scarce and limited to very specific systems, and without application to cellular systems; therefore, it is important to highlight the importance of increasing knowledge in this area. Due to the multiple phenomena that take place at the same time, the mass transfer of CO_2 is difficult to estimate, being influenced by a large number of parameters, such as the physical properties of the gaseous and liquid phase, the operating conditions, the geometric parameters and the state of the biomass. In fermentations, biochemical processes and transport take place at the same time; therefore, it is essential to know the speed at which each one of them takes place and to know the limiting stages. For example, in the event that the rate of transport of the substrates to the cells is higher than the rate of metabolic reactions, the overall rate of conversion would depend on the kinetics of the biochemical reactions. In the opposite case, in which the transfer rate was lower than that of the reaction, it would be essential to make efforts to reduce the average time needed for mass transfer to prevent it from continuing to be the limiting stage. Thus, the study of the overall mechanism of their transfer can be outlined in several stages depending on the location, as shown in Figure 4 [92–95].

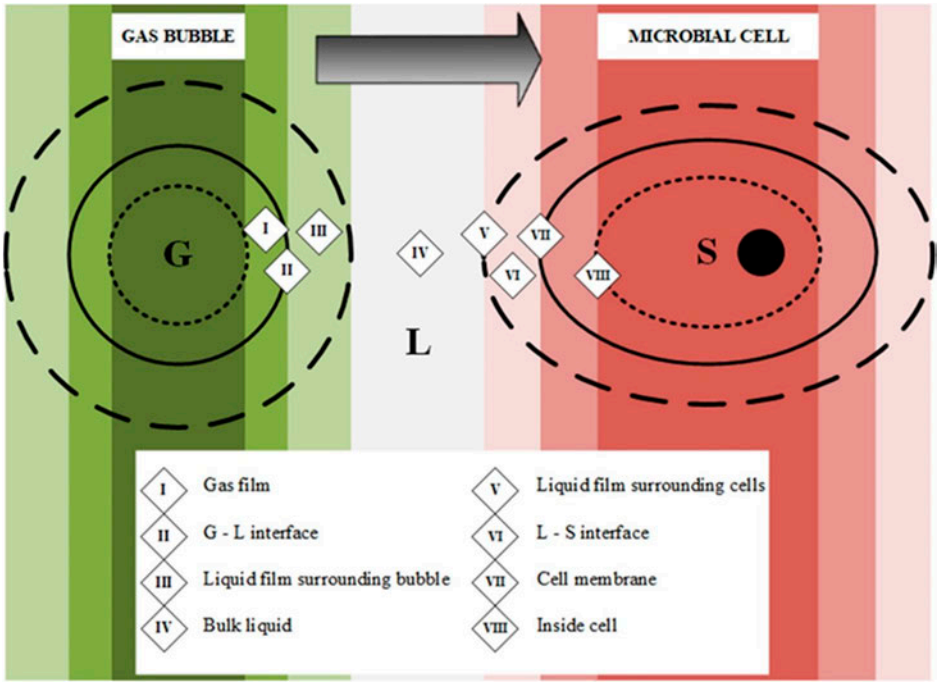

Figure 4. Steps involved in gas transfer from gas bubble to cell.

3.1. Mass Transport in the Liquid Phase

Due to the diffusivities of gases being relatively high compared to those of liquids (103–105 times higher), and their low solubility in water of common gases involved in bioprocesses, the transfer from the gas to the liquid phase is considered the limiting step of the overall process rate [96,97]. In particular, the study of CO_2 transfer is of great importance, since its presence is essential for the activation of the SA production pathway in the TCA cycle [29]. To deepen into this aspect, the following processes should be considered:

3.1.1. CO_2 – Carbonate Equilibrium

When CO_2 dissolves in the fermentation broth, it undergoes a series of chemical equilibrium reactions that follows the model presented below, in which carbonic acid (H_2CO_3), bicarbonate ion (HCO_3^-) and carbonate ion (CO_3^{2-}) are involved [98–101].

$$CO_2 + H_2O \overset{K1}{\leftrightarrow} H_2CO_3 \quad (24)$$

$$H_2CO_3 \overset{K2}{\leftrightarrow} HCO_3^- + H^+ \quad (25)$$

$$HCO_3^- \overset{K3}{\leftrightarrow} H^+ + CO_3^{2-} \quad (26)$$

The equilibrium constants for these three reactions (K_1, K_2, and K_3) can be expressed as follows:

$$K_1 = \frac{[H_2CO_3]}{[CO_2]} \quad (27)$$

$$K_2 = \frac{[HCO_3^-][H^+]}{[H_2CO_3]} \quad (28)$$

$$K_3 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]} \quad (29)$$

Owing to the high instability of carbonic acid in solution and its tendency to dissociate and lose protons, Equations (24) and (25) combine to give Equation (30) [99,100], whose dissociation constant is shown in Equation (31):

$$CO_2 + H_2O \overset{K4}{\leftrightarrow} H^+ + CO_3^{2-} \quad (30)$$

$$K_4 = K_1 \times K_2 = \frac{[HCO_3^-][H^+]}{[CO_2]} \quad (31)$$

Furthermore, it must be taken into account that, in broths with a pH above 7 (and especially those above 8.5), a secondary reaction of hydroxylation of CO_2 with OH^- ions may predominate [102]: however, this equilibrium would not generally be predominant in the production of succinic acid since, as mentioned above, the optimum operating pH is around 6.8.

$$CO_{2\,aq} + OH^- \leftrightarrow HCO_3^- \quad (32)$$

Therefore, the total dissolved CO_2 (C_T) would be the sum of the concentration of CO_2, HCO_3^- and CO_2^- as shown in Equation (33) [98,103,104]:

$$C_{TCO2} = [CO_2] + [HCO_3^-] + [CO_3^{2-}] \quad (33)$$

Considering an approximately neutral pH, the proportion of CO_2 in the total carbonate (α) [98] would be:

$$\alpha = \frac{[CO_2]}{[CO_2] + [HCO_3^-] + [CO_3^{2-}]} \quad (34)$$

If the dissociation constants K_3 and K_4 (with values—at 39 °C—of $K_3 = 6.12\ 10^{-11}$ and $K_4 = 5.35\ 10^{-7}$) are substituted in Equation (34), Equation (35) is obtained, which is a function of the concentration of dissolved protons $[H^+]$ [99,104,105]:

$$\alpha = \frac{1}{1 + \frac{K_4}{[H^+]} + \frac{K_3 \cdot K_4}{[H^+]^2}} \quad (35)$$

From Equations (33) and (35), Equation (36) is derived to obtain the CO_2 concentration [99,104–106]:

$$[CO_2] = \frac{C_T}{1 + \frac{K_4}{[H^+]} + \frac{K_3 \times K_4}{[H^+]^2}} \quad (36)$$

3.1.2. Gas–Liquid Equilibrium

From low to moderate pressures, Henry's law satisfies the ideal equilibrium condition between the liquid and gas phases; thus, Equation (37) can describe the solubility of CO_2 in a pure liquid [98,99,101,105–108]:

$$[CO_2] = \frac{P_{CO_2}}{H_0} \quad (37)$$

where $[CO_2]$ is the concentration of dissolved CO_2 in a liquid (mol·L^{-1}), P_{CO2} is the CO_2 partial pressure in a gas mixture (kPa) and H_0 is the Henry's constant for CO_2 in a pure solvent (kPa m^3 kmol^{-1}).

Due to the fact that a culture medium contains salts and organic substances, it is necessary to describe the solubility of CO_2 using an empirical model such as that suggested by Schumpe and Deckwer—Equation (38). In this model, the salting-out effect of each ion

on a given gas is assumed to be independent from the other ions present in the solution [105,106,108,109]:

$$log\left(\frac{H}{H_0}\right) = log\left(\frac{\alpha_0}{\alpha}\right) = \sum_i (h_i + h_G) \times C_i + \sum_j b_j \times C_{n,j} \quad (38)$$

where H is the Henry's constant in a media, α_0 and α are the Bunsen coefficients to express the CO_2 solubility within a wide temperature range, h is the ion coefficient, h_i (L·mol^{-1}) is the inorganic ion coefficient in the medium (Na^+ = 0.1143; K^+ = 0.0922; Ca^{2+} = 0.1762; Mg^{2+} = 0.1694; H^+ = 0; Cl^- = 0.0318; HPO_4^{2-} = -0.1499; OH^+ = 0.0839; HCO_3^- = 0.0967; CO_3^{2-} = 0.1423)[106], b_j and h_G are estimated by Equation (39) and (40), respectively, as suggested by Weisenberger and Schumpe [110], C_i is the concentration of ion i (mol·L^{-1}) and $C_{n,j}$ is the concentration of organic substance j species (kg m^{-3}).

$$h_G = h_{G,0} + h_{G,T}(T - 298.15) \quad (39)$$

$$b_j = b_n + b_G \quad (40)$$

Here, $h_{G,0}$ for CO_2 is 0.0172 L·mol^{-1} and $h_{G,T}$ is 3.38·10^{-4} L mol^{-1} K^{-1} at temperatures between 273 and 313 K, T is the absolute temperature (K); b_n (m^3 kg^{-1}) for yeast extract is 7.9 10^{-4}, for glucose is 6.68 10^{-4} and for corn steep liquor is 2.11 10^{-4} [98]. b_G can be calculated using the Rischbieter Equation (41) [111]:

$$b_G = b_{G,0} + b_{G,T}(T - 298.15) \quad (41)$$

where $b_{G,0}$ for CO_2 is 1.86·10^{-4} m^3 kg^{-1} and $b_{G,T}$ is 0.01·10^{-4} m^3 kg^{-1} K^{-1} at temperatures between 288 and 323 K [58,59,61].

From Henry's constant H and the partial pressure of CO_2, C_T is obtained with Equation (42) [99,109]:

$$C_{Tco2} = \frac{[CO_2]}{\alpha_0} = \frac{P_{CO_2}}{\alpha_0 \times H} \quad (42)$$

3.1.3. Gas–Liquid Mass Transfer

The transfer of a component from one phase to another is determined by several complex processes including variables such as the interfacial contact area, concentration gradients, molecular diffusivities, mixing conditions, temperature and pressure as well as the rheological phenomena and chemical reactions. The volumetric mass transfer coefficient (k_La) shows the effectiveness of this process considering these variables except for concentration gradients [92,101]; therefore, the carbon dioxide transfer rate (CTR) would be governed by Equation (43) [99,112,113]:

$$CTR = \frac{d[CO_2]}{dt} = k_L a \cdot ([CO_2^*] - [CO_2]) \quad (43)$$

where $[CO_2]$ is the concentration of dissolved CO_2 in the bulk liquid (mol L^{-1}), $[CO_2^*]$ is the saturated CO_2 concentration at a specific CO_2 partial pressure (mol L^{-1}), k_La is in units of reciprocal time and can be estimated with Equation (44) [114]:

$$k_L a = f \times \left(\frac{P_G}{V_w}\right)^a \times v_s^b \quad (44)$$

Here, f is a specific constant related to the geometry of the vessel, P_G/V_w is the gassed power requirement per working volume, V_s is the superficial gas velocity and constants a and b depend on the corresponding correlation.

As explained in the review by Elhajj et al. [101], due to the complexity of the hydrodynamic conditions, a large number of empirical mass transfer correlations have been developed for each system, which are only applicable under very specific conditions.

For well mixed reactors, Hill [115] proposed Equation (45) to calculate the CO_2 transfer, sparged from the bottom of the reactor, in the aqueous phase, not taking into account the bubble size:

$$CTR = \frac{d[CO_2]}{dt} = k_L a \times ([H_2CO_3^*] - [CO_2]) \left(1 - \frac{K_2}{K_2 + (K_2 \times [CO_2]^{0.5})}\right) \quad (45)$$

where $[H_2CO_3^*]$ is the saturation concentration of carbonic acid in aqueous solution and K_2 is the equilibrium constant of the aforementioned equilibrium reaction (25) of the deprotonation of carbonic acid to bicarbonate ion. k_La ranges from 5.6 to 33 10^{-3} s^{-1} and is obtained by Equation (46):

$$k_L a = 33.9 + 6.96 \times \left(\frac{T - 27.5}{7.432}\right) + 15.7 \times \left(\frac{Q_G - 1.1}{0.5351}\right) + 18.8 \times \left(\frac{N - 375}{133.8}\right) \quad (46)$$

in which T is the temperature in ºC, Q_G is the gas flow rate ($L \cdot min^{-1}$) and N is the stirring speed (rpm). This expression is valid under the following conditions: 15 °C < T < 40 °C, 0.2 L min^{-1} < Q_G < 2 L min^{-1}, and 150 rpm < N < 600 rpm.

Because the method originally used for measuring CO_2 solubility and determining k_La was considered incorrect by Kordac and Linek, [101,116], they subsequently proposed Equation (47) as a new expression to estimate *CTR*. In this case, the following assumptions applies: the ideal mixing in the gas phase, negligible resistance to mass transfer in the gas phase, no consideration of the bubble size and the equilibrium reaction of CO_2 in the liquid phase being fast enough to keep carbonate, bicarbonate and hydrogen concentrations in equilibrium at all times.

$$CTR = \frac{d[CO_2]}{dt} = k_L a \cdot \left(\frac{A}{1 + A}\right) ([H_2CO_3^*] - [CO_2]) \left(\frac{2}{2 + \left(\frac{K_2}{[CO_2]}\right)^{0.5}}\right) \quad (47)$$

where A is calculated by Equation (48):

$$A = \frac{Q_G \cdot H}{k_L a \cdot V_w \cdot R \cdot T} \quad (48)$$

where Q_G is the gas flow in L s^{-1} while V_w is the volume of liquid in the reactor (L), R is the ideal gas constant (atm L mol^{-1} K^{-1}) and T is the temperature in K. This correlation is applicable for the following conditions: $0.833 \cdot 10^{-4}$ $m^3 \cdot s^{-1}$ < Q_L < $1.5 \cdot 10^{-4}$ $m^3 \cdot s^{-1}$, 0.583 10^{-3} m^3 s^{-1} < Q_G < 1.17 10^{-3} m^3 s^{-1}, and 0.05 < y_{CO2} <0.2. K_L is a number between 2.06 and 4.69 10^{-3} m s^{-1}.

Equation (49) is proposed to describe the response dynamics of the concentration of carbonic acid in the liquid. This expression can be simplified into Equation (50) assuming that the pH value remains lower than 5 during most of the CO_2 absorption experiments. With the relationship between B and A being established by Equation (51), k_La can finally be solved by Equation (52):

$$\frac{d[H_2CO_3]}{dt} = k_L a \times \frac{A}{1 + A} ([H_2CO_3^*] - [H_2CO_3]) \times \left(\frac{2}{2 + \left(\frac{K_2}{[CO_2]}\right)^{0.5}}\right) \quad (49)$$

$$\frac{d[H_2CO_3]}{dt} = k_L a \times \frac{A}{1+A} 0.99 \times ([H_2CO_3^*] - [H_2CO_3]) = B \times ([H_2CO_3^*] - [H_2CO_3]) \quad (50)$$

$$B = k_L a \times \frac{A}{1+A} \times 0.99 \quad (51)$$

$$k_L a = \left(\frac{0.99}{B} - \frac{V_L \times R \times T}{Q_G \times H}\right)^{-1} \quad (52)$$

3.2. Mass Transport and Biofilm

Low productivity in the biological production of succinic acid is one of the main obstacles to overcome in biorefineries [56,117]. For this, although most of the production studies are carried out in batch or fed-batch operated bioreactors, it is recommended to operate continuously and generate a biofilm, which allows cells to adhere to surfaces at high concentrations, achieving a steady state, non-growing condition and reaching much higher productivities and yields than with other systems [54–56,118]. Furthermore, operation under these conditions promotes the stability and tolerance to toxic substances and inhibitors, because these substances (substrates, products and toxic substances from the raw material hydrolysate, as furfurals or hydroxymethylfurfurals) do not accumulate in the bioreactor over time, diffusing into the biofilm in a restricted way—so their deleterious effect on cells are partly avoided—[119,120]; however, the study of transport phenomena in biofilms poses a great challenge, since they do not have established and well-defined properties. Their structural and physical characteristics such as density, porosity, thickness, cohesion, and cell viability are largely determined by the conditions in which they have been created [119,120]. Unfortunately, all these characteristics and the difficulty to control many of them makes the study of these structures very complex, which has led mostly to the development of equations of a strong empirical character. Biofilm structures are highly irregular with cells showing a tendency to aggregate, thus forming cellular microcolonies of a varying area, shape, and thickness. These microcolonies usually have smaller areas near the surface of the substrate, while the area increases towards the top of the biofilm. This sometimes leads to their fusion, generating even larger microcolonies [121]; therefore, it is logical that the average effective diffusivity (D_e) is dependent on the density, as expressed by Equation (53)[122], and varies along the biofilm—Equations (54) and (55) [123]:

$$D_e = D\left(1 - \frac{0.43 \times \rho_b^{0.92}}{11.19 + 0.27 \times \rho_b^{0.99}}\right) \quad (53)$$

$$D_e = \sum_{n=1}^{p} D_{S,n} \quad (54)$$

$$D_s = 0.25 - 0.95 \times v_L + 2.3 \times [G] + 0.23 \times \left(\frac{z}{Lj}\right) + 0.93 \times v_L \times [G] + 0.59 \times v_L \times \left(\frac{z}{L_b}\right) - 0.7 \times [G] \times \left(\frac{z}{L_b}\right) \quad (55)$$

where ϱ_b is the average density of the biofilm ($kg \cdot m^{-3}$), D is the molecular diffusivity of the reactive species in the medium ($m^2 \cdot s^{-1}$), D_s is the surface averaged relative effective diffusivity, p is the number of measurement points of surface average relative effective diffusivity (vertically), v_L is the velocity in the bulk liquid ($m \cdot s^{-1}$), [G] is the glucose concentration ($kg \cdot m^{-3}$), z is the distance from the bottom of the biofilm (m) and L_b is the average biofilm thickness.

Mokwatlo et al. [124], using Equations (56)–(58), estimated the constant $D_{e\text{-}j}$, which means the effective diffusion constant of compound j in the biofilm as related to the aqueous diffusion constant:

$$\frac{D_{eo-j}}{D_{aq-j}} = \varepsilon_W \times \left(\frac{\varepsilon_{EPS}}{D_{pr}} + \varepsilon_W\right)^{-1} \quad (56)$$

$$\frac{D_{e-j}}{D_{aq-j}} = \left(\frac{D_{e-j}}{D_{eo-j}}\right) \times \left(\frac{D_{eo-j}}{D_{aq-j}}\right) \quad (57)$$

$$\frac{D_{e-j}}{D_{eo-j}} = \left(\frac{\frac{2}{D_{cr}} + \frac{D_{aq-j}}{D_{eo-j}} - 2 \times \varepsilon_{cells} \cdot \left(\frac{1}{D_{cr}} - \frac{D_{aq-j}}{D_{eo-j}}\right)}{\frac{2}{D_{cr}} + \frac{D_{aq-j}}{D_{eo-j}} + \varepsilon_{cells} \times \left(\frac{1}{D_{cr}} - \frac{D_{aq-j}}{D_{eo-j}}\right)}\right) \quad (58)$$

where $D_{aq\text{-}j}$ is the diffusion constant of j in water ($m^2 \cdot s^{-1}$), $D_{eo\text{-}j}$ the effective diffusion constant of j in "cell free" extracellular polymeric substances/water matrix ($m^2 \cdot s^{-1}$), ε_{cells} the biofilm volume fractions of the cells, ε_{EPS} the biofilm volume fractions of extracellular polymeric substances y, and ε_W the biofilm volume fractions of water.

To relate convective mass transfer and mass diffusivity, Horn and Hempel proposed an empirical Equation (59) to calculate the Sherwood number (Sh) [125]:

$$Sh = 2 \times Re^{0.5} \times Sc^{0.5} \times \left(\frac{d}{L}\right)^{0.5} \cdot (1 + 0.0021 \times Re) \quad (59)$$

where Re is the Reynolds number, Sc is the Schmidt number, d is the diameter of the reactor and L is the length of the reactor.

Wäsche et al. [126] accounted for the influence of the biofilm structure on mass transfer, for which they introduced a structure factor (Ω) in the expression of the Sherwood number. Furthermore, they distinguished expressions for the calculation of this number in the laminar regime—Equation (60)—and the turbulent regime—Equation (61).

$$Sh_{laminar} = 2 \times Re^{0.5} \times Sc^{0.5} \times \left(\frac{d}{L}\right)^{0.5} \times \Omega^{-1} \quad (60)$$

$$Sh_{turbulent} = 0.16 \times Re^{0.75} \times Sc^{0.5} \times \Omega^{-1} \quad (61)$$

The structure factor depends on the hydrodynamic and substrate conditions during the biofilm culture, as shown in Equation (62), and it is a function of the Reynolds number as defined in Equation (63):

$$\Omega = 6^{6 \times \mu^*} \times Re_{growth}^{-\left(\frac{\mu^*}{1.5}\right)} \quad (62)$$

$$Re_{growth} = \frac{w \times d}{\nu} \quad (63)$$

where μ^* is the relative growth rate, i.e., the ratio between the specific growth rate at the biofilm surface and the maximum growth rate, w is the mean flow velocity ($m \cdot s^{-1}$) and ν is the kinematic viscosity of water ($m^2 \cdot s^{-1}$).

4. Coupling Dynamic Phenomena to Explain Succinic Acid Production

As challenging as it may be, a comprehensive study of the behavior of the species present in a fermentation broth must jointly consider reaction kinetics and transport phenomena; however, the complexity of these models has meant that their analysis and development is limited and only a couple of studies have appeared in this regard. The combination of dynamic phenomena and kinetic models allows complete information on the

system to be obtained, as mentioned above, being a fundamental tool when optimally designing the reactor, scaling-up, carrying out technical-economic studies and developing a system of control. In addition, knowledge about the optimal flow of CO_2 and the way to achieve an adequate transfer, allows to reduce the costs associated with the feeding of this gas (for example, insufflation expenses, agitation and the capture of excess gas); therefore, in order to achieve the biological production of succinic acid in a sustainable manner, combining the techno-economic and environmental perspectives, the development of knowledge in this area is essential.

Galaction et al. [127] proposed equations to estimate the concentration and flux of glucose as a substrate in a stirred bed of immobilized *A. succinogenes* cells on alginate. For this purpose, they proposed a kinetic model in the mass balance of glucose in the biocatalyst including inhibition by a substrate and product of the Jerusalimsky type. As further assumptions to the model, they assumed steady state, spherical biocatalyst particles, no interactions between the substrate, product and support, and internal diffusion described according to Fick's law and effective diffusivity. From these constraints, Equation (64) is derived, from which Equations (67) and (68) are obtained, taking into account the boundary conditions—Equations (65) and (66):

$$\frac{d[P]}{dt} = \frac{D \times [P]}{[X]} \tag{64}$$

$$\frac{d^2[S]_P}{dr^2} + \frac{2}{r} \times \frac{d[S]_P}{dr} = \frac{v_{max} \times [X]}{D_e} \times \left(\frac{K_{IS}}{K_{IS} + [S]_P}\right) \times \left(\frac{K_{IP}}{K_{IP} + Y_{P/S} \times [S]_P}\right) \tag{65}$$

$$\text{s.t.: } r = 0 \ \rightarrow \ \frac{d[S]_P}{dr} = 0 \tag{66}$$

$$\text{s.t.: } r = R_P \ \rightarrow \ -D_e \cdot \frac{d[S]_P}{dr} = k_L \times ([S]_L - [S]_S) \tag{67}$$

$$[S]_P = \frac{Bi \times ([S]_L - [S]_S) \times cosh(3 \times \varphi \times R_P)}{R_P^2} \times \left[\frac{3 \times \varphi}{R_P} - R_P \times tanh(3 \times \varphi \times R_P)\right] \times \frac{sinh(3 \times \varphi \times r)}{r} \tag{68}$$

where r is the radius of the particle, $[S]_P$ is the concentration of substrate within the biocatalyst particle, v_{max} is the maximum reaction rate, $[X]$ is the cell concentration, $[S]_L$ is the concentration in the liquid bulk, $[S]_s$ is the concentration on the surface of the biocatalyst particle, Bi is the Biot number, φ is the Thiele modulus and k_L represents the mass transfer coefficient in the boundary layer at the particle surface.

The substrate flux from the liquid phase to the particle surface (n_L) is described by Equation (69) and the internal mass flow (n_P) can be obtained by combining Fick's law with Equation (67), thereby obtaining Equation (70):

$$n_L = k_L \times ([S]_L - [S]_i) \tag{69}$$

$$n_P = D_e \frac{Bi \times ([S]_L - [S]_i) \times cosh(3 \times \varphi \times R_P)}{R_P^2} \times [3 \times \varphi - R_P \times tanh(3 \times \varphi \times R_P)] \times \left[\frac{3 \times \varphi \times cosh\left(\frac{3 \times \varphi \times r}{R_P}\right)}{R_P \times r} \frac{sinh(3 \times \varphi \times r)}{r^2}\right] \tag{70}$$

From these expressions, it was estimated that the inhibition is more pronounced in the smallest particles, whilst in the larger particles, internal diffusion is the main limiting

step. In fact, in some internal regions of the particle it is possible to reach such low values of the flux that they are considered "biologically inactive regions", with a magnitude varying from 0 to 5.53% of the total volume of particles.

Later, the same authors [128] expanded this study, performing fermentation in a bioreactor with a stationary basket bed of immobilized *A. succinogenes* cells on alginate. In their study, they found that the values of the external mass flows were about 1.4 to 14 times lower than those obtained for the mobile bed, the difference being more important as the biocatalyst particle size increased and the cylindrical-bed thickness decreased. In this case, the biologically inactive region could be even higher, with its magnitude varying between 0.24% and 44% from the overall volume of each biocatalyst particle size studied and being found mostly in the largest particles on the outer surface of the bed of the basket. In addition to the equations used in their previous article, they included the reduction factor (λ). With a similar nature to the classical effectiveness factor (η), λ represents the ratio between the rates of biochemical reaction in heterogeneous and homogeneous systems. Considering Equation (64), the reduction factor can be expressed by Equation (71):

$$\lambda = \frac{3 \times k_L \times ([S]_L - [S]_i) \times cosh(3 \times \varphi \times R_P)}{R_P^4 \times v_{max} \times [C]} \times \frac{\left[\frac{3 \times \varphi}{R_P} - R_P \times tanh(3 \times \varphi \times R_P)\right] \times cosh(3 \times \varphi) \times [3 \times \varphi - tanh(3 \times \varphi)]}{\left(\frac{K_{IS}}{K_{IS} + [S]_P}\right) \times \left(\frac{K_{IP}}{K_{IP} + Y_{P/S} \times [S]_P}\right)} \quad (71)$$

Although these balances include glucose transport as a substrate and reaction kinetics, they fail to include CO_2 transport, an essential molecule for the activation of the metabolic pathway for the production of SA. Rigaki et al. [114] proposed a mechanistic double substrate model to describe the fermentation by *A. succinogenes* of glycerol in batch systems saturated with CO_2. With this model, it is possible to predict the effect of changes in the initial concentrations of glycerol and $MgCO_3$ on the production and consumption rates of the species present in the broth. The evolution of biomass correlates with Equation (72), which is based on the Monod model—Equation (4)—a Luong-type product inhibition—Equation (14)—and also includes a transport term that includes the mass transfer coefficient (k_L):

$$\frac{d[X]}{dt} = \mu_m \times \left(\frac{[Gly]}{K_{Gly} + [Gly]}\right) \times \left(\frac{[CO_2]}{K_{CO_2} + [CO_2]}\right) \times \left(1 - \frac{[P]}{[P]_m}\right)^{\beta} - k_L \times [X] \quad (72)$$

The concentrations of the products evolve according to Equation (20) and the concentrations of the substrates (glycerol and CO_2) are predicted by means of Equation (19), but in the case of CO_2, the CTR—Equation (43)—is added to this expression, obtaining Equation (73). Considering turbulent mixing and a low viscosity fermentation medium, k_La fits the empirical expression (74). The expressions of the terms involved in this equation are shown in Equations (75) and (76):

$$\frac{d[CO_2]}{dt} = \left(-\frac{1}{Y_{X[CO_2]}}\right)\frac{d[X]}{dt} + \left(-\sum_i \frac{1}{Y_{Pi}[CO_2]}\right)\frac{dP_i}{dt} + (-m)[X] + k_L a \times ([CO_2^*] - [CO_2]) \quad (73)$$

$$k_L a = (2.0 + 2.8 \cdot N) \times \left(\frac{P_g}{V_w}\right)^{0,77} \times v_s^{0,67} \quad (74)$$

$$P_G = q \cdot \left(\frac{P_o^2 \times N \times d^3}{Q^{0.56}} \right)^{0.45} \quad (75)$$

$$P_o = 0.035 \times \mu \times N^3 \times d^{3.7} \times W \times B^{0.8} \times R^{0.4} \times J^{0.3} \quad (76)$$

where P_g is the gas power requirement, V_w the working volume, v_s the superficial gas velocity, q is the impeller type, d the reactor diameter, Q the volumetric flow rate, Po the power requirement for non-gassed Newtonian fluids, B the number of blades, W their width, R the number of baffles, J their width and μ the dynamic viscosity of the medium.

5. Conclusions

Succinic acid has emerged as a very interesting biobased product that could play a pivotal role in the future of biorefineries due to its applications and representing a building block to a wide array of products. This piece of work has summarized and presented systematically the available information on the kinetic modelling of fermentations to this valuable product in the open literature.

The study of a process focused on its implementation at an industrial level, and, therefore, of a bioprocess, requires an approach from the perspective of chemical engineering. In this review, the existing information on the succinic acid production process by biotechnological means has been gathered, focusing on the mathematical models used by different authors for the description of all the physical and chemical (or biochemical) phenomena involved in the overall rate of the process. The scarcity of global models capable of simulating the complex behavior of the system is highlighted.

There have been good attempts at describing fermentations with microorganisms that are known to perform them. In addition, overall, the models developed are also scarce from the point of view of the still relatively low variety of substrates employed in the studies. In general, the proposed kinetic models are very restricted, being mostly non-segregated and unstructured without considering in any case the effects of hydrodynamic stress. Moreover, due to the complexity not only of the chemical reaction network but also the set of mass transports involved, the great challenge of combining transport phenomena with a kinetic model in the bioprocess still remains largely unsolved.

The publications are scarce and provide partial information, focusing on the resistance of the solid substrate through the biofilm or on the transport of CO_2. Whilst all of this in principle represents a complication towards an accurate numerical description and prediction of the process, it also opens an opportunity for researchers in the field and the development of experimental and numerical methodologies. The development of kinetic models and their coupling to equations describing transport phenomena are essential to take into account, together with the mass and heat balances relevant to the bioreactor of interest, when it comes to a detailed design control and operation of the bioreactor, as the most critical unit within any bioprocess. This is key to reducing the operating costs through techno-economical optimization and guides the scaling-up of prototypes and control systems.

Abbreviations

AA	Acetic Acid
ADP	Adenosine DiPhosphate
ATP	Adenosine TriPhosphate
CTR	Carbon dioxide Transfer Rate
C4	4 Carbon containing compound
C5	5 Carbon containing compound
C6	6 Carbon containing compound
ET	Ethanol

FAD+	Flavin Adenine Dinocluotide
FA	Formic Acid
Fructose 1, 6 – P	Fructose 1, 6 – Phosphate
Fructose 6 – P	Fructose 6 – Phosphate
GDP	Gross Domestic Product
Glucose 6 – P	Glucose 6 Phosphate
Glyceraldehyde 3 – P	Glyceraldehyde 3 – Phosphate
IB	Integrated Biorefineries
LA	Lactic Acid
NADH	Nicotinamide Adenine Dinucleotide reduced
NAD+	Nicotinamide Adenine Dinucleotide
PA	Pyruvic Acid
PEP	Phosphoenol Pyruvate
PEPC	Phosphoenol Pyruvate Carboxylase
PEPCK	Phosphoenol Pyruvate Carboxykinase
PYC	Pyruvate Carboxylase
TCA	TriCarboxylic Acid Cycle
TRL	Technology Readiness Level
Ribose 5 – P	Ribose 5 – Phosphate
Ribulose 5 – P	Ribulose 5 – Phosphate
ROS	Reactive Oxygen Species
R+D+I	Research, Development and Innovation
SA	Succinic Acid
Sedoheptulose 7 – P	Sedoheptulose 7 – Phosphate
US DOE	United States Department of Energy
Xylulose 5 – P	Xylulose 5 – Phosphate
a	volumetric coefficient (m^3), exponent in Equation (21).
b	Schenov constant of organic substances ($m^3 \cdot kg^{-1}$), exponent in Equation (21)
B	number of blades
Bi	Biot number
C	concentration ($mol \cdot L^{-1}$, $kg\ L^{-1}$)
d	diameter of the reactor (m)
D	diffusivity ($m^2\ s^{-1}$), dilution rate ($g\ L^{-1}$)
D_e	effective diffusivity ($m^2\ s^{-1}$)
E	Euler number
f	specific constant related to the geometry of the vessel
h	Schenov constant of salts ($L\ mol^{-1}$)
H_0	Henry's constant for CO_2 in a pure solvent ($kPa\ m^3\ kmol^{-1}$)
J	width of baffles (m)
k	mass transfer coefficient ($m\ s^{-1}$)
K	equilibrium constants, kinetic constants ($g\ L^{-1}$)
L	biofilm thickness, length of the reactor (m)
m	Pirt's coefficient (s^{-1})
n	number of species, substrate flux ($g\ m\ L^{-1}\ s^{-1}$), exponent of Equation (42)
N	stirring speed (rpm)
p	number of measurement points of surface average relative effective diffusivity
P	partial pressure in a gas mixture (kPa), power input under gassed conditions (W)

q	impeller type
Q	flow (L s^{-1}, L min^{-1})
r	particle radius (m)
R	ideal gas constant (atm L mol^{-1} K^{-1}), rate of metabolite production (g L^{-1} h^{-1}), number of baffles
Re	Reynolds number
Sc	Schmidt number
Sh	Sherwood number
t	time (s, min, h)
T	temperature (°C, K)
v	gas velocity, velocity in the bulk liquid (m s^{-1})
V	volume of the liquid (L, m^3)
w	average flow velocity (m s^{-1})
W	width of blades (m)
Y	yield (g g^{-1})
Z	distance from the bottom of the biofilm (m)
$[CO_2]$	concentration of dissolved CO_2 in the bulk liquid (mol L^{-1})
$[CO_3^{2-}]$	concentration of carbonate ion (mol L^{-1})
[G]	glucose concentration (kg m^{-3})
$[H_2CO_3]$	carbonic acid concentration (mol L^{-1})
$[HCO_3^-]$	bicarbonate ion concentration (mol L^{-1})
[P]	product concentration (g L^{-1})
[S]	substrate concentration (g L^{-1})
[X]	biomass concentration (g L^{-1})
α	solubility of CO_2, associated growth parameter in production generation models (g g^{-1}), exponent in Equation (48).
β	non-associated growth parameter in production generation models (g g^{-1} h^{-1}), exponent of Equation (51).
δ	associated growth parameter in substrate consumption models (g g^{-1})
γ	associated growth parameter in substrate consumption models (g g^{-1} h^{-1})
η	effectiveness factor
φ	Thiele modulus
λ	time of the latency phase (h), reduction factor
μ	specific growth rate (s^{-1}, min^{-1}, h^{-1}), dynamic viscosity (kg m^{-1} s^{-1})
ν	reaction rate (kg kg^{-1} s^{-1}), kinematic viscosity (m^2 s^{-1})
ϱ	density (kg m^{-3})
Ω	structure factor
aq	refers to aqueous
b	refers to biofilm
cells	refers to cells in biofilm
EPS	refers to extracellular polymeric substances
G	refers to gas
i	refers to ion I, to species i
IS	refers to inhibition per substrate
j	refers to species j
L	refers to bulk liquid
m	refers to maximum state
n	refers to organic substances
o	refers to non-gassed Newtonian fluids, refers to ``cell free´´ extracellular polymeric substances/water matrix

P	refers to product, biocatalyst particle
s	refers to superficial gas, refers to the surface of the biofilm, refers to the surface of the biocatalyst particle
S	refers to substrate
TCO2	refers to total CO_2
T	refers to a specific temperature
w	refers to working volume, refers to water in biofilm
0	refers to a pure solvent or ambient conditions
1	refers to Equation 1
2	refers to Equation 2
3	refers to Equation 3
4	refers to Equation 4
*	refers to a relative rate, refers to a saturation concentration

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8.2 PUBLICACIÓN 2

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Resumen:

El ácido succínico es un químico de plataforma clave los procesos de fabricación bioquímicos modernos. La optimización de la producción de ácido succínico en términos de concentración y economía de los recursos es de vital importancia. En este trabajo se ha estudiado la producción de ácido succínico mediante el uso de *A. succinogenes* tanto en estado de crecimiento como de reposo. Primero, se comprobó que el proceso catalizado por las células en *resting* fue posible gracias al desarrollo de una estrategia de inóculo de dos pasos y adaptación bacteriana a la fuente de carbono. El proceso se realizó en ausencia de fuente de nitrógeno, impidiendo la multiplicación celular y restringiendo la respiración endógena. Si bien los rendimientos del producto fueron casi idénticos cuando se usaron células en crecimiento (0,44 $g \cdot g^{-1}$) y células en estado de reposo (0,43 $g \cdot g^{-1}$), la formación de subproductos se redujo drásticamente cuando se operó con células en *resting*. Después, se estudió el estado de las células en *resting*, observándose que las células del *biofilm* eran más activas que las que se encontraban en suspensión en términos de actividad específica, pero la menor concentración celular en el *biofilm* afectó negativamente las concentraciones finales de ácido. Finalmente, se propuso un modelo cinético completo y simplificado y se ajustó con éxito a los datos experimentales de concentración de biomasa, sustrato, productos y subproductos, tanto en producciones con células en crecimiento como en *resting*. Estos resultados allanan el camino para la optimización de los procesos de producción de ácido succínico con un consumo reducido de la fuente de nitrógeno, promoviendo una mayor selectividad al ácido objetivo, lo que facilita las operaciones posteriores de separación.

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ORIGINAL ARTICLE

On the succinic acid production from xylose by growing and resting cells of *Actinobacillus succinogenes*: a comparison

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Abstract
Succinic acid is a key platform chemical in modern biochemical manufacturing. Optimization of the production of succinic acid production in terms of product titers and economy of resources is of vital importance. In this work, we have studied the succinic acid production by using both produced using growing and resting cells of *Actinobacillus succinogenes*. First, the process catalyzed by resting cells was enabled by the development of a two-step inoculum strategy which was crucial for the bacterial adaptation to the carbon source. The process was performed in the absence of nitrogen source, disabling cell duplication, and restricting endogenous respiration. While the product yields were almost identical when using growing cells (0.44 $g \cdot g^{-1}$) and cells in resting state (0.43 $g \cdot g^{-1}$); very interestingly, the by-product formation was dramatically reduced when operating with resting cells. Next, the format of resting cells was studied; biofilms were found to be more active than cells in suspension, in terms of specific activity, but the lower cellular concentration in biofilms affected negatively the acid final titers. Finally, a complete and simplified kinetic model was proposed and successfully fitted to the relevant retrieved data of biomass, substrate, products, and by-products both in production with growing and resting cells. These results pave the way for the optimization of succinic acid production processes with reduced nitrogen source consumption, promoting a higher selectivity to the target acid, which facilitates the subsequent downstream separation operations.

Keywords Succinic acid · Resting cells · Kinetic model · *Actinobacillus succinogenes* · Xylose · Nitrogen source

1 Introduction

The current social concern for the environment has boosted research to fulfill the vision of an industry based on environmentally friendly processes that transform biological resources into fuels, chemicals, materials, and food and feed ingredients. These are created through processes, materials, intermediates, and final products constituting the so-called integrated biorefinery [1, 2]. Lignocellulosic biomass represents around 70–95% of the total biomass: it is the most abundant, inexpensive, and renewable raw material [3].

The wall of the plant cells in lignocellulosic biomass is made up of cellulose microfibrils added together to form larger fibers; in turn, these are joined by hemicellulose. Lignin covers both polymers, providing a structural integrity of notable strength and resilience [4, 5].

By subjecting lignocellulosic biomass to hydrolytic processes, hemicelluloses are transformed into their monomers, being xylan a most abundant component of hemicellulose. This xylan polymer constitutes up to half of the grassy biomass and up to 25% of the woody one, so xylose is the second most abundant sugar in lignocellulosic biomass [6], second only to glucose and a very suitable chemical platform for the production through biological processes of value-added products, such as succinic acid [7, 8].

Succinic acid is a key chemical platform and a very promising compound for the bioeconomy era. In 2019, the global market for this compound was valued at $137.4 million with expectations that it will grow 58% by 2026 [9]. Succinic acid is an acidifier, flavoring, and sweetener in the food industry, while it is converted by chemical industry processes into several products: polybutylene succinate, polyesters, and polyols, to name a few. It is also widely used in the pharmaceutical industry, as well as for the production of resins, coatings, and pigments. Recently,

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its potential to manufacture biodegradable polymers has been highlighted [10–12].

Succinic acid is as an intermediate of the tricarboxylic acid cycle, so it can be produced by a wide variety of fungi and bacteria. However, bacteria isolated from the rumen of ruminants are the most promising candidates [13], including *Actinobacillus succinogenes* due to its ability to naturally use CO_2 to produce high amounts of this acid in anaerobic conditions [14, 15]. Furthermore, this microorganism is capable of using xylose as a carbon source with high efficiency through the pentose phosphate pathway [6, 16].

One of the limiting factors in the biological production of this compound is the high cost of the nitrogen source. Ventrone et al. (2020) [17] observed that high C:N ratios could have a favorable impact on succinic acid production by decreasing by-product formation. However, to our best knowledge, to date, there are no studies on the production of succinic acid in the absence of a source of nitrogen, a situation that induces the resting state in cells.

Cell-catalyzed biotransformations can take place during cell growth, but also when cells are in a resting state. Cells in a resting state do not experience growth; however, they are metabolically active. This situation permits to separate the process into a biocatalyst production stage and the biotransformation itself. Filtration and centrifugation allow separating the growth biomass obtained in the first stage, a biocatalyst that is subsequently used in the production stage, suspended in a medium composed exclusively of the carbon source, and a buffer solution that allows maintaining osmotic pressure conditions. In general, the objective of a cell-catalyzed operation in a resting state is to direct the metabolism towards the product of interest while minimizing the generation of by-products [18, 19], since high-cost purification techniques are one of the main disadvantages of the bioproduction of succinic acid [20]. Another notable advantage of operating with cells in a resting state in the independent optimization of the growth and production stages is being able to avoid inhibitory phenomena due to substrates and products associated with cell growth [18, 19, 21].

In view of the literature, the possibility of using a resting state biocatalyst for succinic acid production is completely innovative. Therefore, the objective of this research is to study the feasibility of producing succinic acid from xylose in the absence of a source of nitrogen using *A. succinogenes* as a biocatalyst. This verification would open doors to numerous studies aimed at minimizing production costs: (i) carrying out multi-stage productions with cells in a resting state starting from a single batch of growth cells; (ii) running production processes with resting-state cells created in an starting batch of growing and reactivating them from production batch to production batch; and (iii) defining novel strategies to separate cells for resting-cell production while minimizing mechanical stresses, to name a few possibilities [22–24].

In this work, yields and productivities in processes run with growing and resting cells are compared, after a preliminary study on the influence of the presence or absence of an adaptation stage to the carbon source and the addition of a pre-inoculum stage. An additional objective is to determine the differences in activity between free and immobilized cells in the resting state. Finally, a complete but simple kinetic model is proposed and fitted with high accuracy to biomass, substrate, products, and by-product data for both growing and resting cells.

2 Materials and methods

2.1 Bacterial strain, adaptation, preinoculum, and inoculum stages

Actinobacillus succinogenes DSM 22,257 was supplied by the German Collection of Microorganisms and Cell Cultures GmbH. To prepare the microorganism, we distributed the complete content of the lyophilisate in the ampoule on one agar plate with Brain Heart Infusion (BHI) and incubated at 37 °C for 2 days. For the long-term preservation, the strain was incubated in Tryptic Soy Broth (TSB) at 37 °C for 1 day; then, a mixture of the culture with glycerol (1:1 v/v) was prepared and stored in 1-mL Eppendorf tubes at − 80 °C.

To study the effect of the microorganism adaptation to the carbon source, the thawed cells were inoculated in 100-mL bottles containing 60 mL of TSB purged with N_2 at a flow rate of 1 L min^{-1} for 2 min. The serum bottles were incubated at 37 °C in orbital shaker at 200 rpm for 24 h. Subsequently, adding 5% (v/v) of growing cells, a second growth was performed in which the medium was supplemented with 10 g L^{-1} of xylose. After 24 h, a final growth step was performed increasing the xylose concentration to 20 g L^{-1} and adding 5% (v/v) of cells from the 10 g L^{-1} xylose culture. The preinoculum and inoculum stages were carried out in bottles under the same conditions as the last stage of adaptation. In the case of the preinoculum, 10% (v/v) of the culture adapted to 20 g L^{-1} of xylose was inoculated, while in the case of the inoculum, 10% (v/v) of the previous preinoculum culture was used. $NaHCO_3$ was added into the medium in a concentration equal to that of the carbohydrate.

2.2 Succinic acid production in a growing cells reactor

The experiments were performed in a 2-L stirred tank bioreactor (STBR) BIOSTAT B-Plus (Sartorius AG, Germany) with a working volume of 1 L. All runs were conducted with TSB and 20 g L^{-1} of xylose. Fermentations were operated

at 37 °C with mixing at 300 rpm. The pH was controlled at 6.8 by automatic addition of NaOH 5 M. Carbon dioxide was sparged with a flow rate 0.1 vvm.

To determine the inoculation volume, biomass concentrations were measured by UV–vis spectrophotometry in the exponential phase of the pre-inoculum and the inoculum. Precisely, due to the study of the use of pre-inocula and inocula (which may or may not have been adapted to sugar), the inoculation to the reactor can be carried out from cultures with different concentrations of biomass. To avoid this issue, an initial concentration of biomass is fixed, instead of an inoculation percentage, with the aim of normalizing experiments. Based on the evolution of the biomass concentration over time in pre-inocula and inocula, we decided to operate with an initial concentration of 0.05 g L^{-1} of biomass, since this allowed to inoculate broths of preinocula and inocula in exponential phase without exceeding in any case 10% (v/v) of the culture medium of the reactor. Runs were performed in triplicate.

2.3 Succinic acid production in a resting cells reactor

After 15 h, 30 h, or 40 h of fermentation in the growing cell reactor, free or suspended biomass was separated from the liquid broth by centrifugation and washed with a K_2HPO_4 solution. Subsequently, the biomass was transferred to a second 1 L STBR with 0.5 L of working volume to carry out production. This second stage was performed employing as medium a buffered solution of xylose: K_2HPO_4 50 mM supplemented with 20 g L^{-1} xylose at 37 °C and 300 rpm. The pH was controlled at 6.8 by automatic addition of NaOH 5 M. Carbon dioxide was supplied at 0.05 vvm flow rate to the liquid medium.

In those cases in which biofilm had appeared in the growing cells reactor, runs were performed with immobilized resting cells of the biofilm. To do this, liquid broth was drained through the sampling tube, taking care not to damage the biofilm adhered to the walls of the reactor. Then, through the sampling tube, the reactor was filled again but this time with resting medium, K_2HPO_4 50 mM and 20 g L^{-1} of xylose. In this way, fermentation was carried out under the same conditions as when starting from centrifuged cells, but starting exclusively from the cells present in the biofilm. Runs were performed in triplicate.

2.4 Analytical methods

The concentration of biomass was measured by UV–vis spectrophotometry using a Shimadzu UV–vis spectrophotometer UV-1603, obtaining the optical density data at 600 nm.

Substrate and product concentrations were analyzed by high-performance liquid chromatography (HPLC) using a refractive index detector (RID) at 55 °C, by means of an Agilent Technologies 100 series equipment. A REZEX ROA-Monosaccharide H^+ (8%) column (300 × 7.8 mm, Phenomenex, USA) worked at 80 °C employing a H_2SO_4 5 mM solution as mobile phase at a flow rate of 0.5 mL min^{-1}.

To determine the amount of biomass contained in the growth reactor biofilm, a dry weight of the total amount of the reactor was carried out at the end of the fermentation.

2.5 Mathematical methods

The kinetic model has been based on the evolution of biomass, xylose, and succinic, lactic, formic, and acetic acids.

To fit the model to the experimental data, Aspen Custom Modeler v11 (AspenTech, USA) was employed. An implicit Euler method coupled to a non-linear least-square solver algorithm (NL2SOL) was employed to integrate the differential equations of the kinetic model and to fit the model to the relevant data. The NL2SOL algorithm estimates the parameters by minimizing the difference between experimental and kinetic model simulation data applying the least square method.

Equations (1), (2), and (3) show the statistical parameters that were taken into consideration to evaluate the goodness of fit of the model: Fisher's F-value (F), which should be higher than its tabulated value at 95% confidence to fulfill the null hypothesis, the sum of squared residuals (SSR), and the residual mean squared error (RMSE), which should be as close to zero as possible, and the variation explained (VE), which should be equal or near 100% to indicate an identical change of experimental and simulated data with time.

$$F = \frac{\sum_{i=1}^{N}\left(\frac{y_{i,calc}}{K}\right)^2}{\sum_{i=1}^{N}\left(\frac{SSR}{N-K}\right)} \tag{1}$$

$$RMSE = \sqrt{\frac{SSR}{N-K}} \tag{2}$$

$$VE(\%) = 100\left(1 - \frac{\sum_{l=1}^{L} SSQ_l}{\sum_{l=1}^{L} SSQ_{mean_l}}\right) \tag{3}$$

where N is the total number of experimental data, K is the number of parameters, and SSR is the squared sum of residues, $(y_{i,exp} - y_{i,calc})^2$: $y_{i,exp}$ are the experimental values of the variable, and $y_{i,calc}$ are the calculated values. SSQ_l is the sum of the quadratic residues and SSQ_{meanl} is the squared sum of deviations between the experimental and the mean score with respect to the calculated values.

To quantify the succinic acid production, the maximum yield of succinic acid as a function of the initial xylose

concentration, as a function of xylose, as a function of the maximum concentration of biomass, and the productivity of succinic acid have been defined respectively in Eqs. (4), (5), (6), and (7).

$$Y_{SA/Xyl.0} = \frac{C_{SA.max}}{C_{Xyl.0}} \tag{4}$$

$$Y_{SA/Xyl.cons} = \frac{C_{SA.max}}{C_{Xyl.cons}} \tag{5}$$

$$Y_{SA/X} = \frac{C_{SA.max}}{C_{X.max}} \tag{6}$$

$$P_{SA} = \frac{C_{SA.max}}{time} \tag{7}$$

where $C_{SA,max}$ is the is the maximum concentration of succinic acid, $C_{Xyl.0}$ is the initial concentration of xylose, $C_{Xyl.cons}$ is the concentration of xylose consumed and $C_{X.max}$ is the maximum concentration of biomass.

3 Results

3.1 Influence of adaptation on preinoculum and inoculum stages

The evolution of the biomass concentration (C_X) over time in the preinoculum and inoculum stages is shown in Fig. 1, where the effect of the prior adaptation to sugar on the production concentration in the reactor can be noticed. This figure shows that the use of two stages (preinoculum–inoculum) allows for an increase in biomass production rate and final biomass concentration. This fact suggests that, as it is common in industrial practice, the inoculum must be created through more than one stage, increasing the viability of the microorganism from one stage to the next. Thanks to the successive stages of growth, an excessive cell density at the beginning of the process can be avoided, as the small concentration of viable cells adapts easily to the production medium with enough space and nutrients to proliferate while preventing apoptosis or programmed cell death from taking place [25].

3.2 Influence of adaptation and inoculum on the production of succinic acid with growing cells

In order to compare the performance of cells in the growing and resting state, four runs have been carried out in bioreactors whose production medium contains a nitrogen source. Figure 2 shows the consumption of xylose (C_{XYL}) and production of biomass, succinic acid (C_{SA}) and by-products (C_{SP}) for four

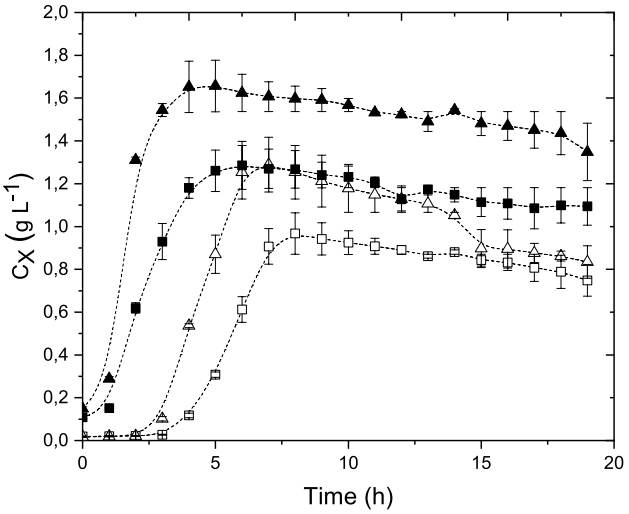

Fig. 1 Effect of adaptation on biomass growth in the preinoculum and inoculum stages in flasks. Data points: preinoculum without adaptation (□), preinoculum with adaptation (△), inoculum without adaptation (■), and inoculum with adaptation (▲)

runs performed under identical batch conditions starting from 20 g L^{-1} xylose with the cells in a growing state. The differences between them lay in performing (or not) an adaptation of the strain to xylose and in the number of inoculum stages (one or two).

The progressive adaptation of the microorganism to xylose during the inoculation and pre-inoculation stages facilitates its viability (more biomass in less time and more biomass in the steady state) and affects its productivities towards succinic acid and the accompanying by-products (acetic acid and formic acid, mainly). With two inoculation stages (pre-inoculation + inoculation) before the production stage, there is an increase in biomass, as well as in the production of succinic acid and by-products. Furthermore, when adaptation is performed, xylose is totally consumed when starting from a relatively low concentration (20 g L^{-1}). Thus, it is possible to hypothesize that, in the absence of progressive adaptation to xylose, the metabolic steps leading to succinic acid are deactivated or strongly inhibited. In general, these phenomena also affect by-product yields, whose final concentration would decrease from 5 to 1 g L^{-1}. In Table 1, there is a short compilation of all key fermentation parameters obtained in the absence and the presence of a progressive adaptation to xylose and the use of one or two inoculum stages.

3.3 Influence of adaptation and inocula on the production of succinic acid with resting cells

A. succinogenes is able to produce succinic acid after the cell growth has stopped. In fact, according to Werf et al. (2016), the rate of succinic acid production remains constant before

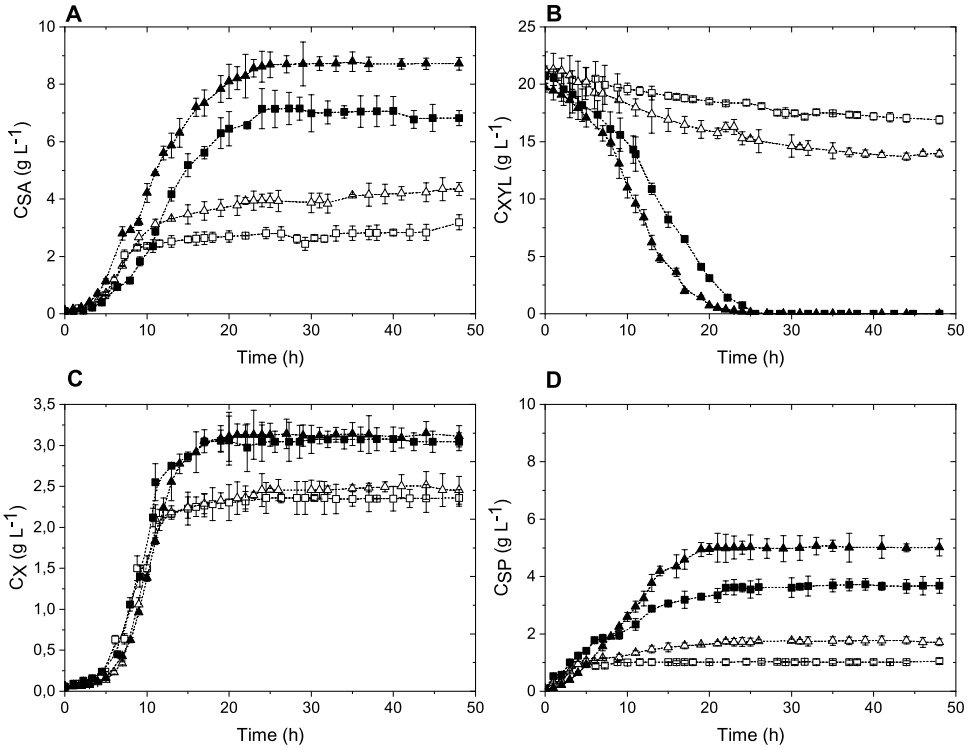

Fig. 2 Effect of adaptation and preinoculum stage on succinic acid production (**A**), xylose consumption (**B**), biomass growth (**C**), and by-products production (**D**) during fermentation with growing cells in bioreactor. Data points: no adaptation and no preinoculum stage (□), no adaptation and preinoculum stage (△), adaptation and no preinoculum stage (■), adaptation and preinoculum stage (▲)

Table 1 Concentrations, selectivity, yields and productivities on succinic acid production with growing and resting cells depending on adaptation and preinoculum stage

	Growing cells				Resting cells			
Run	1	2	3	4	5	6	7	8
Adaptation	No	No	Yes	Yes	No	No	Yes	Yes
Preinoculum	No	Yes	No	Yes	No	Yes	No	Yes
C_{SA} (g_{SA} L^{-1})	3.19	5.83	6.82	8.72	0.28	0.80	2.30	8.51
C_{SA} / C_{SP} (g_{SA} g_{SP}^{-1})	3.02	3.40	1.86	1.74		5.70	2.63	2.97
$Y_{SA/Xyl.0}$ (g_{SA} $g_{Xyl.0}^{-1}$)	0.15	0.27	0.33	0.44	0.01	0.04	0.11	0.43
$Y_{SA/Xyl.cons}$ (g_{SA} $g_{Xyl.cons}^{-1}$)	0.78	0.79	0.33	0.44	1.14	0.53	0.65	0.81
$Y_{SA/X}$ (g_{SA} g_{X}^{-1})	1.35	2.37	2.24	2.80	0.08	0.24	0.56	2.03
P_{SA} (g_{SA} L^{-1} h^{-1})	0.11	0.22	0.30	0.36	0.01	0.04	0.12	0.18

and after the end of the exponential growth phase [26]. This is a key point if bacteria in the resting state are to be used as biocatalyst.

In this study, the production of succinic acid has been carried out in reactors in which cell reproduction is stopped due to the absence of a source of nitrogen. These cells are inoculated in high concentration, coming from reactors whose culture medium has a source of nitrogen and the salts required for the growth of the microorganism. Figure 3 shows the evolution of the species involved in a batch fermentation with cells in a resting state starting from 20 g L^{-1} xylose.

Although the production of succinic acid with cells in growing state is favored by adapting the microorganism to sugar and adding a pre-inoculum stage, it can be observed that, in the case of using cells in resting state, these stages are essential. When using resting cells, two consecutive inoculum stages lead to a more productive microorganism. However, a progressive adaptation of the strain to xylose is even more important. The addition of this step practically increases from three to eight times the production of succinic acid. As these cells are subjected to great hydrodynamic stress due to the centrifugation and washing process, they clearly require a prior strengthening process so as not to be deactivated in the absence of a nitrogen source. In fact, the production of succinic acid without adaptation just reaches 1 g L^{-1}, stopping the metabolic flow immediately. In the case of performing an adaptation, but not a pre-inoculum stage, the growth trend in the early stages (up to 6 h) is similar to the case in which one more inoculum stage is added. Nevertheless, from this moment,

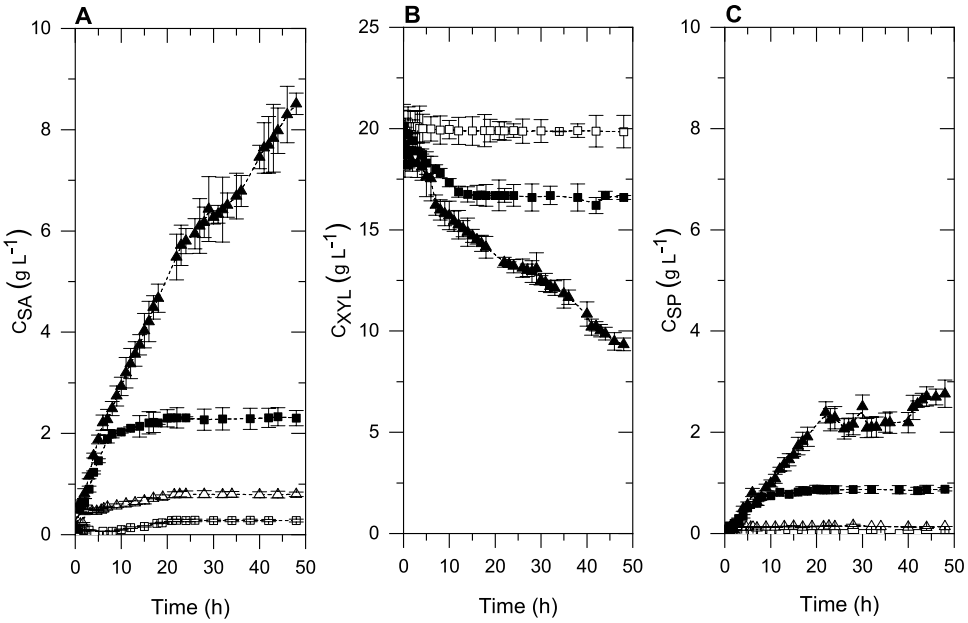

Fig. 3 Effect of adaptation and preinoculum stage on succinic acid production (**A**), xylose consumption (**B**), by-products production (**C**) during fermentation with resting cells. Data points: no adaptation and no preinoculum stage (□), no adaptation and preinoculum stage (△), adaptation and no preinoculum stage (■), adaptation and preinoculum stage (▲)

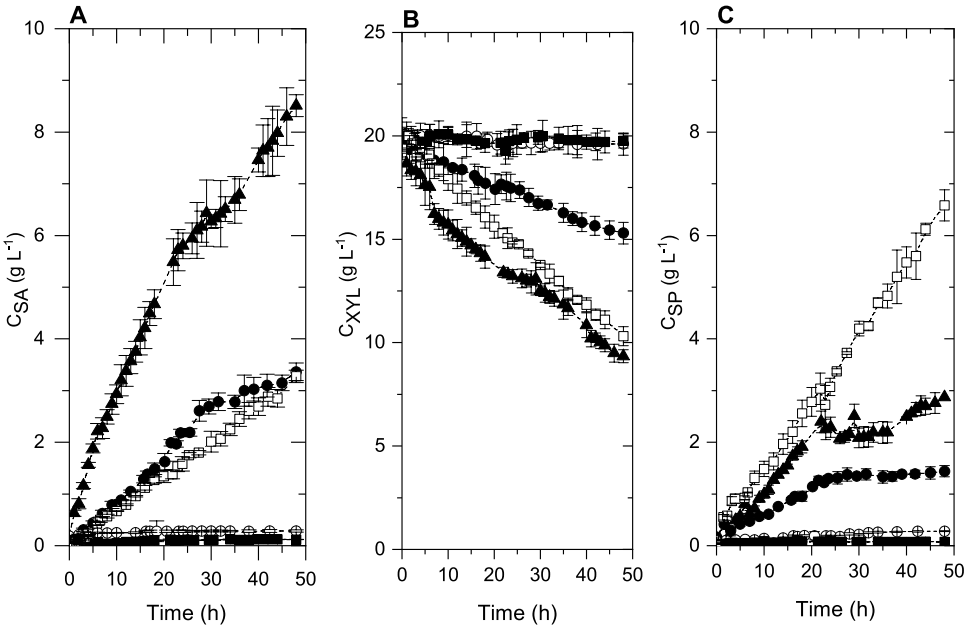

Fig. 4 Effect of age and cell status on succinic acid production (**A**), xylose consumption (**B**), by-products production (**C**) during fermentation with resting cells. Data points: free cells from the end of the exponential phase (▲), free cells in the early stages of biofilm formation (●), cells immobilized in the biofilm in the early stages of biofilm formation (○), free cells in advanced times of biofilm formation (■), cells immobilized in biofilm at advanced times of biofilm formation (□)

it undergoes a deactivation, preventing the concentration of succinic acid from rising beyond 2.3 g L^{-1}. A similar evolution can be observed in the generation of by-products, the value becoming stable at 0.8 g L^{-1}.

The growth of succinic acid in the case of having adaptation and preinoculum stage reaches a value of 8.51 g L^{-1}, similar to that obtained using cells in a growing state (8.72 g L^{-1}). Although the production rate with resting cells is lower than that of growing cells, at 48 h production, it is still increasing, with 9.3 g L^{-1} of xylose remaining in the broth. Therefore, it can be easily deduced that, at more advanced times and/or higher initial biomass, the yield would reach higher values. However, in fermentation with growing cells at 24 h, the xylose has been completely consumed and the maximum performance of the process has been reached, without the possibility of increasing this value except by changing the operating mode to fed-batch.

Most curiously, the pathways towards acetic acid and formic are deactivated around 10 h in the case of operating with resting cells obtained in a process with adaptation and

preinoculum stage while the rate of generation of succinic acid remains constant during 48 h. From this time onwards, the consumption of xylose is directed almost exclusively to the generation of succinic acid, which would allow to achieve much higher yields at more advanced times. All key fermentation results when operating with resting cells in and the presence of a progressive adaptation to xylose and the use of one or two inocula stages are compiled in Table 1.

3.4 Importance of age and cell status on the production of succinic acid with resting cells

A. succinogenes is naturally capable of forming biofilms, so the cells are embedded in a self-produced matrix of extracellular polymeric substances in gel form [27]. This occurs at long operating times, self-adhering to the support surfaces [28]. Therefore, we wanted to verify the feasibility of operating with cells immobilized in the biofilm in the absence of a source of nitrogen and to compare their performance with those corresponding to growing cells, as shown in Fig. 1.

In reactors whose medium contains the nutrients for cell reproduction, that is, those used as inoculum for reactors with cells in a resting state, at 15 h, there are only free cells in the broth. However, at 30 h, there is a small amount of biofilm adhered to the walls of the reactor and at 40 h, this matrix has increased considerably and does not seem to increase its size any further. Therefore, the experiments were carried out from the cells suspended in the broth at 15 h, from the free cells and from the immobilized cells, separately, in a reactor after 30 h of fermentation and again, from free cells and immobilized cells but after 40 h of fermentation (Fig. 4).

The highest succinic acid productions were reached from free cells at 15 h. The productions carried out from free cells at 30 h of fermentation obtained the following best results, as well as in the case in which immobilized cells were used after 40 h of operation. It should be noted that, in the three fermentations, the succinic acid production rate remains practically constant over time, with a considerable amount of xylose still in the broth at 48 h, which suggests that, unlike those fermentations carried out in the presence of a source of nitrogen (see Fig. 2), at later times of fermentation, higher yields could have been achieved. However, in the case of operating from immobilized cells and free cells after 30 h and 40 h of fermentation respectively, the production of succinic acid is negligible. Table 2 shows the comparison of the final concentrations of succinic acid, yields, and productivities of these experiments, as well as the concentrations of the inoculated biomass in each of the reactors ($C_{X.0}$).

Table 3 shows a compilation of the experiments carried out according to the presence of the adaptation stage, preinoculum stage, state, and cell age.

Table 2 Concentrations, selectivity, yields and productivities on succinic acid production with resting cells depending on age and cell status

Run	8	9	10	11	12
Time growth reactor (h)	15	30	30	40	40
Cells state	Free	Free	Biofilm	Free	Biofilm
C_{SA} (g_{SA} L^{-1})	8.51	3.36	0.29	0.12	3.28
C_{SA} / C_{SP} (g_{SA} g_{SP}^{-1})	2.97	2.34	1.02	1.57	0.50
$Y_{SA/Xyl.0}$ (g_{SA} $g_{Xyl.0}^{-1}$)	0.43	0.17	0.01	0.01	0.17
$Y_{SA/Xyl.cons}$ (g_{SA} $g_{Xyl.cons}^{-1}$)	0.81	0.72	0.62	1.11	0.36
$Y_{SA/X}$ (g_{SA} g_{X}^{-1})	2.03	0.80	3.96	0.03	21.9
$C_{X.0}$ (g_{X0} L^{-1})	4.20	4.14	0.07	3.78	0.15
P_{SA} (g_{SA} L^{-1} h^{-1})	0.18	0.07	0.02	0.01	0.07

3.5 Kinetic modeling of succinic acid production with growing cells versus resting cells

It has already been determined that the best results in batch fermentation were obtained; thanks to carrying out previous stages of adaptation of the microorganism to sugar and adding a pre-inoculum stage, using both cells in growth and resting state. In addition, the production in the absence of a nitrogen source obtained better results when a high concentration of free cells was used after 15 h of fermentation in a reactor whose medium was rich in nutrients.

Since the development of mathematical models that describe the behavior of the species involved in fermentation turns out being a fundamental step to enable industrial implementation [29], a simple non-structured non-segregated kinetic model has been proposed and fitted to all relevant experimental data from runs featuring the best results when using both growing and resting state cells.

3.5.1 Kinetic modeling of succinic acid production with growing cells

The proposed scheme is described in Eqs. (8) and (9) and their associated reactions in Eqs. (10) and (11). This kinetic model suggests that the carbon source (S) is transformed into biomass (X) and by-products (SP), acetic and formic acid mainly represented grouped and in parallel to succinic acid (P). Therefore, the by-product production is associated with growth, while the production of succinic acid is not associated with growth, according to the experimental observations. Equations (12), (13), (14), and (15) show the consumption and production rates of each of the compounds involved.

$$Y_{S,X} \bullet S \rightarrow X + Y_{SP,X} \bullet SP \tag{8}$$

$$Y_{S,P} \bullet S \rightarrow P \tag{9}$$

Table 3 Compilation of experiments depending on age and cells status and the addition of adaptation or preinoculum stages

Run	Cells state		Adaptation	Preinoculum	Time growth reactor (h)
	Growing/Resting	Free/Biofilm			
1	Growing	Free	No	No	-
2	Growing	Free	No	Yes	-
3	Growing	Free	Yes	No	-
4	Growing	Free	Yes	Yes	-
5	Resting	Free	No	No	15
6	Resting	Free	No	Yes	15
7	Resting	Free	Yes	No	15
8	Resting	Free	Yes	Yes	15
9	Resting	Free	Yes	Yes	30
10	Resting	Biofilm	Yes	Yes	30
11	Resting	Free	Yes	Yes	40
12	Resting	Biofilm	Yes	Yes	40

$$r_1 = \mu \bullet C_X \bullet \left(1 - \frac{C_X}{C_{X,m}}\right) \tag{10}$$

$$r_2 = k_p \bullet C_S \bullet C_X \tag{11}$$

$$R_X = r_1 \tag{12}$$

$$R_S = -Y_{S,X} \bullet r_1 - Y_{S,P} \bullet r_2 \tag{13}$$

$$R_P = r_2 \tag{14}$$

$$R_{SP} = Y_{SP,X} \bullet r_1 \tag{15}$$

where $Y_{i,j}$ is the macroscopic yield of compound "i" with respect to compound "j" ($mol_i\ mol_j^{-1}$), r_i is the reaction rate for the reaction "i" ($g\ L^{-1}\ h^{-1}$), μ is the specific growth rate (h^{-1}), k_p is the kinetic constant in the succinic acid production reaction ($g\ L^{-1}$), and R_i is the rate of metabolite "i" production ($g\ L^{-1}\ h^{-1}$).

Figure 5 shows the fit of the values predicted by the model to the experimental data over time and Table 4 shows the estimated kinetic parameters, as well as the statistical parameters that provide information on the goodness of fit.

3.5.2 Kinetic modeling of succinic acid production with resting cells

During a fermentation in the absence of a nitrogen source, cell reproduction is stopped, so the chemical reaction scheme that describes the production of succinic acid and by-products is reduced to a single expression (Eq. (12)) and a single reaction rate associated (Eq. (20)). The consumption and production rates are shown in Eqs. (16), (17), (18), and (19).

Figure 6 shows the result of the adjustment made in relation to the experimental data. The kinetic and statistical parameters obtained are presented in Table 5.

$$Y_{S,P} \bullet S \rightarrow P + Y_{SP,P} \bullet SP \tag{16}$$

$$r_1 = k_p \bullet C_S \bullet C_X \tag{17}$$

$$R_S = -Y_{S,P} \bullet r_1 \tag{18}$$

$$R_P = r_1 \tag{19}$$

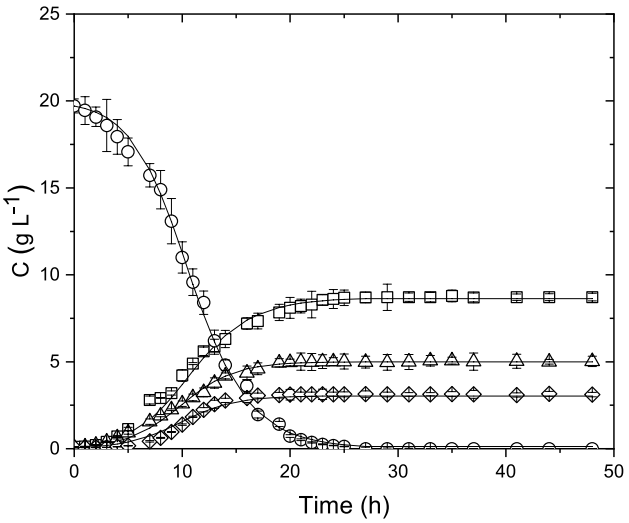

Fig. 5 Kinetic modeling of succinic acid production with growing cells. Data points: observed xylose (○), observed succinic acid (□), observed by-products (△), observed biomass (◇). Model predictions shown as lines

Table 4 Kinetic and statistical parameter values calculated by fitting the kinetic model to experimental data of succinic acid production with growing cell runs

$C_{X,M}$ (g L^{-1})	3.04 ± 0.08	F_{95}	9194
$K_p \times 10^{-2}$ (h L g^{-1})	4.44 ± 0.17	RMSE	0.27
μ (h^{-1})	0.40 ± 0.01	SSR	7.24
$Y_{S,P}$ (g g^{-1})	2.00 ± 0.08	VE (%)	99.5
$Y_{SP,X}$ (g g^{-1})	1.65 ± 0.05		
$Y_{S,X}$ (g g^{-1})	0.85 ± 0.04		

$$R_{SP} = Y_{SP,P} \bullet r_1 \tag{20}$$

4 Discussion

The importance of adapting *Actinobacillus succinogenes* to the carbon source to boost the production of succinic acid is a topic that, until now, has not been studied in depth. However, several authors have considered it relevant to add a pre-inoculum stage in their experimental methodology. This allows for better cell conditioning and greater experimental reproducibility, which is ideal when conducting experiments comparing different operating conditions. For example, Salvachúa et al. included a pre-inoculum stage in their batch succinic acid production process to compare the effect of different hydrolysis processes in corn stover as well as different initial concentrations of glucose and xylose [30]. Another example is the study on the effect of the concentration of

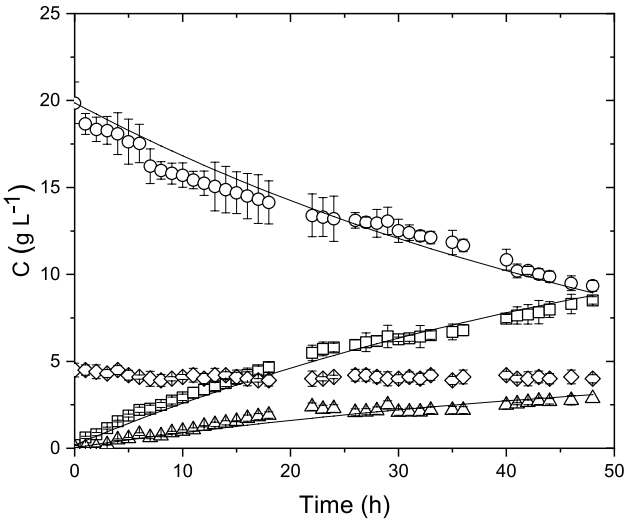

Fig. 6 Kinetic modeling of succinic acid production with resting cells. Data points: observed xylose (○), observed succinic acid (□), observed by-products (△), observed biomass (◊). Model predictions shown as lines

Table 5 Kinetic and statistical parameter values and confidence intervals calculated by fitting the kinetic model to experimental data of succinic acid production with resting cell runs

$K_p \times 10^{-3}$ (h L g^{-1})	3.12 ± 0.05	F_{95}	1320
$Y_{S,P}$ (g g^{-1})	1.27 ± 0.02	RMSE	0.48
$Y_{SP,P}$ (g g^{-1})	0.35 ± 0.02	SSR	27.4
		VE (%)	94.9

dissolved CO_2 and $MgCO_3$ in the succinic acid production process carried out by Zou et al. (2011), who also included a preinoculum stage in the bottle after having grown colonies on plates [31].

In this work, the effect of the adaptation of the microorganism to the carbon source and the addition of a pre-inoculum stage on the production of succinic acid with growing cells has been studied (results corresponding to runs 1 to 4). When considering the final succinic acid titer or concentration as shown in Table 1, it is evident that adaptation and a greater number of inoculum stages play a critical role in obtaining the highest possible value of this parameter. Similar conclusions are withdrawn when considering the yield to succinic acid related to the initial concentration of xylose ($Y_{SA/Xyl.0}$), the yield per unit mass of biomass ($Y_{SA/X}$), or the final productivity to acid (P_{SA}). However, when looking at the acid yield per gram of xylose consumed ($Y_{SA/Xyl.cons}$), adaptation to this monosaccharide appears to be deleterious, suggesting that there is greater selectivity to acid at low acid production (which is the case when no adaptation is made). Furthermore, adaptation appears to play a more important role than the addition of a preinoculum stage. In a fermentation without a preinoculum stage but in which a previous adaptation to xylose is carried out, the final concentration of succinic acid increases 3.63 g L^{-1}, whereas if the microorganism is not adapted but a pre-inoculum stage is carried out, the increase is lower: 2.64 g L^{-1}.

Bearing in mind that in the experiments with resting cells that will be discussed later (runs 5 to 12), batch fermentation with growing cells in the presence of a source of nitrogen was exclusively performed to obtain a high concentration of biomass; the culture medium selected in these experiments with growing cells (runs 1–4) is the one typically employed in the preinoculum and inoculum stages [32–34]. Despite not using an optimal medium for production, in the fermentations carried out with a previous adaptation and a pre-inoculum stage, a yield of 0.44 g g^{-1} and a productivity of 0.36 g L^{-1} h^{-1} have been achieved in this study, values close to those obtained by other authors as shown in Table 6. Recently, Jokodola et al. (2022) studied the use of hemicellulosic fractions rich in xylose from olive pits and sugarcane bagasse as well as pure xylose, with which they obtained a batch yield of 0.25 g g^{-1} and a high productivity of 0.78 g L^{-1} h^{-1} [35]. However, a high amount of by-products was

generated, with the ratio between the concentration of succinic acid and by-products (C_{SA}/C_{SP}) being 54% lower than that of this study. Almqvist et al. (2016) compared the acid production from the monosaccharides xylose, arabinose, glucose, mannose, and galactose, achieving the highest yields and productivities with glucose, being respectively 0.56 g/g and 0.7 g g^{-1}, while with xylose, these values were reduced to 0.42 g g^{-1} and 0.18 g L^{-1} h^{-1} [36]. Ferone et al. (2017) focused on the production of succinic acid using representative sugars from the hydrolysis of lignocellulose (glucose, mannose, arabinose, and xylose), observing synergistic effects of the co-presence of the four sugars, achieving a yield of 0.55 g g^{-1} and a productivity 0.22 g L^{-1} [37]. Bradfield et al. (2016) managed to drastically increase the productivity of succinic acid from xylose operating continuously, reaching values of 3.4 g L^{-1} h^{-1} [38].

After studying the effect of adaptation and the addition of a pre-inoculum stage in fermentation with cells in a growth state, this same study was carried out on the production of succinic acid with cells in a resting state (runs 5 to 8), that is, as explained above, in a medium without nitrogen source.

In Table 1, it can be seen how, in these runs, the final concentrations of succinic acid are 30 times higher by adapting the bacteria to xylose and adding a pre-inoculum stage, while with growing cells, it was only 2.7 times higher. Depending on the number of culture conditioning stages included in the process, yields grow from practically 0to 43% and productivities from 0.01 to 0.18 g L^{-1} h^{-1}. In fact, in a process with adaptation and pre-inoculum stage, the maximum yield achieved in the absence of nitrogen at 48 h is very similar to that obtained with cells in a growing state (0.44 g g^{-1}). Furthermore, as mentioned above, due to the growth trends observed and the amount of xylose remaining in the broth at 48 h, in the case of production with cells in a resting state, this growth would probably continue to increase at longer times. Due to this fact, at 48 h the yield of succinic acid generated with these cells compared to the xylose consumed practically doubles the value corresponding to that obtained during the fermentation process with cells whose growth has not been stopped. However, the productivity of succinic acid with resting cells is significantly lower, being half the value corresponding to the one reached by cells that are growing, which generate most of this acid during the exponential phase of growth.

Since this process starts with a high cell density, in those cases in which it has not been possible to reach a high concentration of succinic acid, the yields in relation to the biomass present in the culture broth are negligible, being 0.08 g g^{-1} and 0.24 g g^{-1} in the cases without adaptation and in the absence and the presence of a pre-inoculum stage, respectively, while those corresponding to cells in a growing state are 1.35 g g^{-1} and 2.37 g g^{-1}. However, once a complete conditioning of the microorganism has been carried out, these values are closer between cells in a resting state (2.03 g g^{-1}) and in a growth state (2.8 g g^{-1}).

It should be noted that, as happened in fermentations driven in the presence of nitrogen source, the selectivity decreases as the succinic yield increases. This means that, under adverse conditions, the predominant metabolic pathway is the generation of succinic acid. This is a plausible explanation of the very high selectivity to succinic acid obtained in the production process with cells in a resting

Table 6 Comparison of succinic acid production with previous works depending on substrate, nitrogen source, and *A. succinogenes* cell state

Cell state		Type of operation	Substrate	Nitrogen source	Yield (g_{SA} $g_{substrate}^{-1}$)	Productivity (g_{SA} L^{-1} h^{-1})	C_{SA} (g_{SA} L^{-1})	Ref
Growing/resting	Free/biofilm							
Growing	Free	Batch	Xylose	Yeast extract	0.25	0.39	18.9	Jokodola et al. (2022) [35]
Growing	Free	Batch	Xylose	Yeast extract	0.42	0.15	3.94	Almqvist et al. (2016) [36]
Growing	Free	Batch	Glucose, mannose, arabinose, xylose	Yeast extract	0.55	0.22	27.0	Ferone et al. (2015) [37]
Growing	Biofilm supported	Continuous	Xylose	Yeast extract, CSL	0.57	3.6	10.7	Bradfield et al. (2016) [6]
Growing	Free	Batch	Chestnut shells	Yeast extract, soy peptone	0.62	0.76	10.0	Ventrone et al. (2020) [17]
Growing	Free	Batch	Glucose	CSL	0.76	0.75	37.9	Xi et al. (2013) [39]
Growing	Free	Batch	Xylose	Yeast extract	0.44	0.36	8.72	This study
Resting	Free	Batch	Xylose	None	0.43	0.18	8.51	This study

state, as they are subjected to the stresses due to centrifugation and the lack of nutrients. In fact, in a process with previous adaptation and two inoculum stages, with resting cells, it is possible to achieve a selectivity 1.7 times higher than the respective process with growing cells. In literature, there are precedents of the negative effect of high nitrogen source concentrations on the selectivity of succinic acid (results shown in Table 6). Ventrone et al. (2020) observed, in the fermentation of chestnut shell hydrolysate, how higher C:N ratios and/or nitrogen limitation promote the biosynthesis of succinic acid to the detriment of biomass and other acid by-products [17]. Xi et al. (2013) used corn steep liquor (CSL) instead of yeast extract and observed that, in the production of succinic from glucose, the relationship between the concentrations of this acid and acetic acid turned out to be 4.57 when the culture medium contained 15 g L^{-1} of CSL, while this ratio increased to 5.25 when the amount of CSL decreased to 7.5 g L^{-1} [39].

After studying the effect of adaptation to the carbon source and the addition of a pre-inoculum stage on both the production of succinic acid with cells in growth and in a resting state, the influence of age and cell status on production with cells in a resting state was also studied. Specifically, the study has focused on the influence of the age and state of the cells obtained in the growth reactor that serve as inoculum for the stage of production of succinic acid without a nitrogen source, that is, with cells in a state of rest. As mentioned in the "Results" section, the effect of inoculating cells in the production reactor at rest after 15 h, 30 h, or 40 h of fermentation has been compared. In addition, since at 30 h, there is already biofilm formation on the walls of the reactor, experiments have been carried out with cells at rest in suspension and immobilized in the biofilm after 30 h and 40 h in the growth reactor.

As can be seen in Table 2, the fermentation in which it has been possible to achieve a higher value of succinic acid yield with respect to the initial xylose concentration (0.43 g g^{-1}) and productivity (0.18 g L^{-1} h^{-1}) is the one in which free cells at 15 h have been used. The experiments in which free cells at 30 h and immobilized cells at 40 h were used led to identical yields and productivities (0.17 g g^{-1} and 0.07 g L^{-1} h^{-1}).

However, the cellular concentration present in the biofilm at 40 h (determined by dry weight) is 28 times lower than that of the fermentation carried out from free cells at 15 h. Therefore, the yields of succinic acid in relation to the biomass of 21.9 g g^{-1} and 2.03 g g^{-1}, respectively, which indicates that the metabolic activity of immobilized cells is higher than those that are suspended in the broth. Mokwatlo et al. (2020) already noted the advantages of operating with biofilm, given that the matrix of extracellular polymeric substances confers on it many beneficial attributes, such as a long-term sustained activity and an improved tolerance to environments that would otherwise be toxic. Furthermore, biofilm bacteria constitute a coordinated functional community that is more efficient than floating planktonic cells, which is made possible thanks to the physiological cooperativity of bacterial cells in stable juxtaposition with cells of the same species. The biofilm is protected from desiccation, gives cells an improved tolerance against antimicrobial agents, and acts as an absorber for nutrients in the liquid phase. In summary, the biofilm way of life provides bacterial cells with emergent characteristics that are superior to those of the suspended way of life [40, 41]. However, high cell density fermentation can be described as a requirement to improve volumetric productivity and subsequently reduce capital expenditures. This requires a cell retention strategy in which cells are separated from the fermentation broth and concentrated in the fermenter [28].

It has also been found in this work that the amount of biofilm in its early stages of formation, that is, at 30 h, is insufficient to carry out a fermentation process. On the other hand, free cells at 40 h have lost metabolic activity since, despite being in high concentration in the broth (3.78 g L^{-1}), the concentration of succinic acid generated is insignificant (0.12 g L^{-1}).

After determining the best conditions of adaptation, age, and cell state, kinetic models were proposed for the fermentations that obtained the best results both with cells in growth (run 4) and with cells in a resting state (run 8).

As shown in Fig. 5, the model fits very reasonably to all relevant data from run 4, that is, from fermentation with growing cells and stages of adaptation and preinoculum. Goodness-of-fit statistical parameters (Table 4) indicate a high value for Fisher's *F*, much over the limiting value (the value of the *F* tabulated at 95% confidence is 3.7), and a low value for the SRC. Likewise, the experimental trend with time and the one retrieved from the model are very similar, so VE percentage is very high.

In literature, we can find numerous non-segregated unstructured models for the fermentation process with *A. succinogenes* but most of these works are limited to describing the evolution of biomass or the main product, succinic acid [37, 42–47]. However, some authors have proposed reaction schemes that contemplate the consumption of the substrate, as well as the generation of biomass, products, and by-products. For the description of the evolution of biomass, Lin et al. (2008) proposed a combination of the Malthus' Law for the exponential phase in steady state systems with Loung's expression for substrate and for product inhibition. Pateraki et al. (2016) and Vlysidis et al. (2008) also introduced the Haldane-Andrews expression with the inhibition constant per substrate. Although Lin et al. (2008) and Pateraki et al. (2016) proposed the same equation to describe the substrate consumption during fermentation, while Vlysidis et al. (2008) considered another expression

more appropriate, in both cases, they introduced the so-called Pirt's maintenance coefficient, considering that part of the substrate is also consumed to maintain biomass in a viable state throughout the reaction. All these authors agreed on the use of the Leudeking-Piret expression to predict the evolution of the concentrations of succinic acid and by-products [45–47].

All these models use complex equations with a high number of parameters that consider the possible inhibition effects that each of the species present in the broth may exert. While these are of course effects that must be taken into consideration when studying a system in detail, they can pose a problem when applied on an industrial scale. One of the strengths of the model proposed in this study that differentiates it from conventional work is that it has minimized the amount of parameters present. This simplification of the reaction scheme and of the kinetic equations, however, has resulted in a very accurate fit of the model to all experimental data, supporting the hypothesis of an absence of complex inhibition phenomena in these experimental conditions, as no further parameters or higher model complexity is needed to achieve an adequate fit.

Despite the differences between the parameters of this model and those previously presented in the literature, certain similarities can be found in the values of some parameters. For example, for this model, a value of 0.4 h^{-1} was estimated at the constant μ. These authors considered μ dependent on the concentration of substrate and product, inversely proportional to inhibition constants, and directly proportional to the maximum specific growth rate (μ_m), being the constant with the greatest weight in these equations. Lin et al. (2008) obtained a value of 0.5 h^{-1} for μ_m of using glucose as a carbon source. This parameter, in the case of Vlysidis et al. (2008), was 0.12 h^{-1} (using glycerol as carbon source) and, in the study by Pateraki et al. (2016), its values reached 0.394 h^{-1} with a mixture of xylose, galactose, glucose mannose, and arabinose as carbon source [45–47].

In this study, using xylose as a carbon source, the parameters $Y_{S,X}$ and $Y_{S,P}$, involved in the substrate consumption rate equation, are respectively 0.85 g g^{-1} and 2.0 g g^{-1}. These values are of the same order of magnitude although slightly lower than those obtained by Vlysidis et al. (2008) (0. 13 g g^{-1} and 2.79 g g^{-1}), who also obtained an insignificant Pirt coefficient (0.001 s^{-1}) [47]. These values are hardly comparable to those of the other authors, since the results they showed were values of the sum of these parameters among themselves and of the sum Y_{PiS} with the Pirt maintenance coefficient [45, 46].

In the model proposed here, the succinic acid production equation is directly proportional to the concentration of substrate, biomass, and the biomass production rate, while the by-product generation rate is only dependent on the latter. Therefore, it is not possible to make a reliable comparison with the results obtained by other authors, since they consider the production of both succinic acid and by-products as a sum of two terms, one associated with biomass growth and the other independent of it [45–47].

Finally, the fit was made for the kinetic model proposed for run 8, that is, the production of succinic acid with resting cells in which free cells from a growth reactor was inoculated after 15 h of fermentation and in whose process included a cell adaptation stage and a preinoculum stage.

Despite the simplicity of the model, a good fit to the experimental data is achieved (Fig. 5), as demonstrated by a high value of Fisher's F (the value of the F tabulated at 95% probability is 8.55) and VE shown in Table 5. However, the accuracy of the fit is not as good as in the case of the model proposed for growing cells (Table 4), whose RMSE and SSR were lower (0.27 and 7.24).

Comparing the kp parameters of both estimates, it can be seen how the model proposed for cells in a resting state predicts a substantially lower succinic acid production rate ($3.12 \cdot 10^{-3}$ $h \cdot L \cdot g^{-1}$) than in the case of fermentation in a medium rich in nutrients ($4.44 \cdot 10^{-2}$ $h \cdot L \cdot g^{-1}$). This fact, together with the lower value of $Y_{S,P}$ (1.27 g g^{-1}) compared to growing cells (2 g g^{-1}), justifies a lower consumption of the carbon source and, therefore, the possible potential to generate an increased amount of succinic acid beyond 48 h of fermentation or at a higher initial cell concentration.

From these parameters, a much greater selectivity can also be deduced in the operation with cells in resting, since the relationship between the parameters $Y_{S,P}$ and $Y_{SP,P}$ in this type of operation is 3.63, while the relationship between $Y_{S,P}$ and $Y_{SP,X}$ is 1.21 operating with cells that continue to reproduce. This should be highlighted as the global atomic economy of the bioprocess based on resting cells, of only considering the production itself, is much higher than when using growing cells. Moreover, the use of resting cells allows to separate the production of the biomass, the biocatalyst, and that of the main chemical product, a good strategy to optimize each of them separately [21, 48].

5 Conclusion

In summary, the possibility of producing succinic acid using *A. succinogenes* in a resting state is demonstrated, while the importance of the bacteria adaptation to the carbon source and the multiple consecutive inocula stages are shown to increase the acid titer, yield, and productivity. Furthermore, the best results were obtained starting from suspended cells at 15 h of growth. If the cell age is higher, yields decrease. When observing immobilized cells in a biofilm behavior, due to their lower concentration in the biosystem, a lower concentration of succinic acid is achieved despite the fact that these cells have a much higher specific activity than the free ones. The kinetics of the bioprocesses driven by

growing and resting cells were assessed with two non-aggregated non-segregated models proposed to describe the evolution of the species involved in the production processes; in both cases, very similar yields to SA were obtained, but the selectivity to the acid is much higher with resting cells. The process developed in this study using bacteria cells under resting conditions could be a powerful approach for the optimization of succinic acid bioprocesses based on a tandem approach: biomass generation followed by acid production.

Author contribution IAE: conceptualization, methodology, investigation, data curation, and writing—original draft. ML and VES: conceptualization, data curation, supervision, writing—review & editing, project administration, and funding acquisition.

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Declarations

Conflict of interest The authors declare no competing interest.

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8.3 PUBLICACIÓN 3

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Resumen:

El ácido succínico (SA) es considerado uno de los principales químicos de plataforma. En este trabajo abordamos el estudio de su producción por *Actinobacillus succinogenes* DSM 22257 a partir de glucosa, centrándonos en el desarrollo y aplicación de un modelo cinético simple capaz de representar la evolución del proceso en el tiempo para una gran diversidad de variables de proceso clave para la producción: concentración de biomasa inicial, concentración de extracto de levadura, velocidad de agitación y caudal de dióxido de carbono. Todas estas variables han sido estudiadas experimentalmente, determinando los valores de los parámetros clave de la fermentación: concentración (23,8-39,7 $g \cdot L^{-1}$), rendimiento (0,59-0,72 $g_{SA} \cdot g_{glu}^{-1}$), productividad (0,48-0,96 $g_{SA} \cdot L^{-1} \cdot h^{-1}$) y selectividad (0,61-0,69 $g_{SA} \cdot g_{glu}^{-1}$). Incluso con esta amplia diversidad de condiciones operativas, un modelo cinético no estructurado y no segregado fue capaz de ajustarse a los datos experimentales con alta precisión, considerando los valores de los parámetros estadísticos de bondad de ajuste. Este modelo se basa en la ecuación logística para el crecimiento de biomasa y en ecuaciones cinéticas potenciales para describir la evolución de SA y la suma de subproductos como no asociados al crecimiento de biomasa. La aplicación del modelo cinético bajo diversas condiciones operativas arrojó luz sobre su efecto en la producción de SA: parece que el estrés por déficit de nitrógeno es una buena condición para la concentración y la selectividad de SA, existe un volúmen de inóculo óptimo para este propósito y el estrés hidrodinámico comienza a las 300 r.p.m. Debido a su importancia práctica, y para validar el modelo cinético desarrollado, también se llevó a cabo una fermentación tipo *fed-batch*, comprobando la bondad del modelo propuesto a través de la simulación del proceso (etapa o ciclo 1) y aplicación a ciclos posteriores de la alimentación, determinando que la inactivación de la biomasa comenzó en el ciclo 3.

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Development of a Simple and Robust Kinetic Model for the Production of Succinic Acid from Glucose Depending on Different Operating Conditions

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Abstract: Succinic acid (SA) is one of the main identified biomass-derived chemical building blocks. In this work we approach the study of its production by *Actinobacillus succinogenes* DSM 22257 from glucose, focusing on the development and application of a simple kinetic model capable of representing the evolution of the process over time for a great diversity of process variables key to the production of this platform bio-based chemical: initial biomass concentration, yeast extract concentration, agitation speed, and carbon dioxide flow rate. All these variables were studied experimentally, determining the values of key fermentation parameters: titer (23.8–39.7 $g \cdot L^{-1}$), yield (0.59–0.72 $g_{SA} \cdot g_{glu}^{-1}$), productivity (0.48–0.96 $g_{SA} \cdot L^{-1} \cdot h^{-1}$), and selectivity (0.61–0.69 $g_{SA} \cdot g_{glu}^{-1}$). Even with this wide diversity of operational conditions, a non-structured and non-segregated kinetic model was suitable for fitting to experimental data with high accuracy, considering the values of the goodness-of-fit statistical parameters. This model is based on the logistic equation for biomass growth and on potential kinetic equations to describe the evolution of SA and the sum of by-products as production events that are not associated with biomass growth. The application of the kinetic model to diverse operational conditions sheds light on their effect on SA production. It seems that nitrogen stress is a good condition for SA titer and selectivity, there is an optimal inoculum mass for this purpose, and hydrodynamic stress starts at 300 r.p.m. in the experimental set-up employed. Due to its practical importance, and to validate the developed kinetic model, a fed-batch fermentation was also carried out, verifying the goodness of the model proposed via the process simulation (stage or cycle 1) and application to further cycles of the fed-batch operation. The results showed that biomass inactivation started at cycle 3 after a grace period in cycle 2.

Keywords: succinic acid; fermentation; kinetic model; operational conditions; carbon dioxide

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1. Introduction

The growing concern about the effects of climate change and the depletion of fossil resource reserves have driven a movement focused on the use of new renewable energy sources and bio-based chemicals [1]. One of the most promising alternatives is the development of biorefineries, where biomass will be sustainably processed into bioproducts (materials and chemicals) and bioenergy (biofuels, electricity, and heat) [2].

Within the top 12 high-value-added chemicals from biomass, according to the US Department of Energy (DOE) [3], succinic acid is considered one of the key carboxylic acids for the bioeconomy era. Although this compound is widely used in the production of polyesters, polyols, resins, coatings, and pigments, and in the pharmaceutical and food industries, in the context of biorefineries, its main applications are the generation of intermediate chemical products such as 1,4-butanediol, tetrahydrofuran, 2-pyrrolidine, or maleic acid. Furthermore, the potential application of succinic acid and its derivatives for the manufacture of biodegradable polymers should also be mentioned [4–6].

Currently, the production volume of succinic acid through the biological route has already exceeded that generated by traditional chemical production. In addition, this growth is expected to continue in the coming years, so that the biotechnological production of this compound will go from generating a market value of USD 170 million in 2020 up to USD 2.22 billion by 2026 [7].

Among the microorganisms that produce succinic acid, it is worth highlighting *Actinobacillus succinogenes*, isolated from the rumen, as one of the most promising bacteria, since this acid is a final product during its anaerobic metabolism [8,9].

Numerous studies have been carried out focused on determining the influence of different variables on the production process of succinic acid using this microorganism as a biocatalyst. Despite the initial concentration of substrate being one of the most studied operating conditions, there is not yet a scientific consensus on its optimal value. For example, in the experiments carried out by Luthfi et al. [10] and Salvachúa et al. [11], the highest yields were achieved in fermentations starting from a concentration of 60 g L^{-1} of glucose, while Ferone et al. [12] achieved the best results when this concentration was reduced to 40 g L^{-1}. These latter authors also observed that low concentrations of xylose, around 5 g L^{-1}, increased the yield of succinic generation. However, Pateraki et al. [13], when using a mixture of sugars rich in xylose as a carbon source, achieved better results at a total sugar concentration of 32.5 g L^{-1}.

Another of the most studied parameters is the CO_2 source. Diverse authors have employed different carbonates ($NaHCO_3$, K_2CO_3, $CaCO_3$, or $MgCO_3$) at several concentrations [14,15], comparing them with pure gaseous CO_2 or biogas at different partial pressures [16–19], and even maximizing their solubility by increasing the pressure in the reactor [20,21]. Other studies have focused on cell status and on the mode of operation, performing experiments with free, immobilized cells or resting cells in batch, repeated batch, fed-batch, and continuous mode operations [22–28]. It is worth noting the work of Kim et al. [23], who carried out a continuous fermentation of recycled cells to maximize the biocatalytic activity, achieving a productivity of 3.86 g L^{-1} h^{-1} of succinic acid. However, the yields and productivities are especially high when working with immobilized cells, operating both in repeated batch. For example, Cao et al. [27] increased the production of succinic acid by approximately 50% compared to the batch operation. Furthermore, in continuous mode, Ercole et al. [25] produced 36.5 g L^{-1} h^{-1} of succinic acid with cells entrapped in alginate beads.

However, there is a lack of knowledge on other conditions that also affect the fermentation process with this microorganism, such as the initial biomass concentration or the agitation speed. In addition, until now, studies focused on the nitrogen source are scarce and the impact that this variable may have on production has not been fully explored [16,29,30]. It is worth noting the work of Tan et al. [31], who compared the use of 15 g L^{-1} of yeast extract (YE) and corn steep liquor (CSL), achieving a succinic acid amount 3.7 % lower than using yeast extract as the nitrogen source and reducing by a fifth the costs associated with the nitrogen source. Xi et al. [32] studied the effect of using CSL at different initial concentrations, obtaining similar yields in fermentations with CSL and YE as long as the amount of CSL doubled that of YE. Jiang et al. [33] successfully replaced the yeast extract with a spent brewer's yeast hydrolysate with vitamin supplements.

Besides the many studies and proofs of concept published for the development of biorefinery processes, the main drawback of most of the proposed concepts is their viability at industrial scale. To carry out the scaling of a fermentation process, it is essential to develop kinetic models with equations capable of predicting the behavior of the species involved throughout production. These models allow an adequate selection of the type of operation, as well as the optimal design and operation of the bioreactor. In addition, they are very useful for the implementation of the control system and the performance of techno-economic studies [7,34].

However, the state-of-the-art research shows that the kinetic models developed on succinic acid production are based on results of fermentations carried out at different

initial concentrations of substrate. Therefore, as these studies are empirical in nature, they are only valid for the particular experimental conditions tested. Furthermore, there are no kinetic studies taking into account the effects of other operating conditions that are as influential as the concentration of the main carbon source. For these reasons, this work goes beyond the state-of-the-art research, using a novel approach to accomplish an exhaustive study of the influence exerted by diverse key operation variables—the initial concentration of biomass, the speed of agitation, and the concentration of yeast extract—in a batch production of succinic acid from glucose through the action of *A. succinogenes*. The developed new kinetic model is simple but capable of predicting the evolution of the concentration of biomass, glucose, succinic acid, and by-products for each of the experiments, and it was also applied to the production stages of a fed-batch fermentation.

2. Materials and Methods

2.1. Microorganism

Actinobacillus succinogenes DSM 22257, supplied by the German Collection of Microorganisms and Cell Cultures GmbH, was used in all experiments.

2.2. Culture Media

For storage, a 1:1 *v*/*v* glycerol/ Tryptic Soy Broth (TSB) mixture was used [15,23,35] and for its subsequent reactivation, only TSB was used. TSB composition was (in grams per liter): 17 tryptone, 3 soytone, 2.5 glucose, 5 NaCl, 2.5 K_2HPO_4.

The medium for inoculum preparation as well as for the production reactor was the same [36] (in grams per liter): 3 K_2HPO_4, 0.43 $MgCl_2.6H2O$, 0.2 $CaCl_2$, 1 NaCl, 40 glucose, 2.5 / 5 / 7.5 / 10 yeast extract. In the case of the inoculum, 40 g L^{-1} of $NaHCO_3$ was added as a CO_2 source and for pH control. Carbon and nitrogen sources were autoclaved separately.

2.3. Cultivation Conditions

First, the stored cells were thawed at −80 °C and injected into bottles with 60 mL of TSB, which were previously purged with N_2 for 2 minutes at a flow rate of 1 L min^{-1}. After incubating the bottles at 37 °C and 200 rpm for 24 h, the inoculum was grown under the same conditions, but using, in this case, the production medium.

The study of the influence of operating conditions was carried out in a 2 L stirred tank bioreactor (STBR) BIOSTAT B-Plus (Sartorius AG, Germany) with a working volume of 1 L. In all runs, 0.1 vvm CO_2 was bubbled into the broth while operation was performed at 37 °C and a pH of 6.8 controlled by automatic addition of 5 M NaOH. The production medium was (in grams per liter): 3 K_2HPO_4, 0.43 $MgCl_2.6H2O$, 0.2 $CaCl_2$, 1 NaCl, 40 glucose, 2.5 / 5 / 7.5 / 10 yeast extract. Experiments were carried out at different stirring speeds: 150, 200, 250, and 300 rpm, as well as at three different initial biomass concentrations: 0.05, 0.075, and 0.1 g L^{-1}.

In the fed-batch-type operation, the fermentation was carried out in the same reactor and with the same culture medium as in the batch type operations, working at 37 °C, 300 rpm, and pH 6.8 (5M NaOH), with an initial biomass concentration of 0.05 g L^{-1} and a yeast extract concentration of 10 g L^{-1}. After the first stage, a concentrated glucose solution was fed at the start of each of the following stages.

2.4. Analytical Methods

A spectrophotometer (Shimadzu UV-vis spectrophotometer UV-1603) was used to measure the biomass concentration, obtaining optical density data at 600 nm.

Glucose, succinic acid, and by-products (formic and acetic acids) were quantified by high-performance liquid chromatography (HPLC) (Agilent Technologies 100 series). The column employed for this analysis was a REZEX ROA-Monosaccharide H+ (8%) column

(300 × 7.8 mm, Phenomenex, USA) at 80 °C pumping a H_2SO_4 5 mM solution as mobile phase at a flow rate of 0.5 mL min^{-1}. The refraction index detector worked at 55 °C.

3. Results and Discussion

The results for succinic acid titer (C_{SA}), yield with respect to the initial concentration of the carbon source (Y_{SA}), productivity (P_{SA}), and selectivity (S_{SA}) of the batch experiments carried out under different conditions of CO_2 flow, agitation speed, and yeast extract and initial biomass concentrations are shown in Table 1.

It should be noted that the first experiment was taken as the reference one, because its operating conditions are those that can be considered standard or intermediate between those more used in batch-type fermentation for the production of succinic acid using *A. succinogenes* as a biocatalyst [10,11,13,23,26,37–42].

In runs 1, 2, and 3, the initial concentration of biomass is a factor whose variation mainly affected the productivity of the process. It was observed that at an initial concentration of 0.075 g L^{-1}, the intermediate value of those studied, the productivity of succinic acid reaches a maximum value of 0.96 g L^{-1} h^{-1}. However, if the cell concentration is further increased to 0.1 g L^{-1}, productivity is reduced by 21%. In addition, a slight increase in selectivity was observed at higher initial amounts of biomass in the reactor.

Table 1. Summary of succinic acid titers, yields, productivities, and selectivity values under different operational conditions. The first run is the reference one.

Run	Type of operation	$C_{biomass}$ (g·L^{-1})	Agitation (rpm)	CO_2 flow (L·min^{-1})	C_{YE} (g·L^{-1})	C_{SA} (g·L^{-1})	Y_{SA} (g·g^{-1})	P_{SA} (g·L^{-1}·h^{-1})	S_{SA} (g·g^{-1})
1	Batch	0.05	300	0.1	10	27.4	0.68	0.83	0.62
2	Batch	0.075	300	0.1	10	28.5	0.71	0.96	0.64
3	Batch	0.1	300	0.1	10	28.3	0.70	0.76	0.66
4	Batch	0.05	300	0.5	10	27.6	0.69	0.84	0.63
5	Batch	0.05	300	1	10	26.1	0.65	0.81	0.63
6	Batch	0.05	150	0.1	10	23.6	0.59	0.72	0.61
7	Batch	0.05	200	0.1	10	26.4	0.66	0.78	0.62
8	Batch	0.05	250	0.1	10	28.5	0.71	0.84	0.62
9	Batch	0.05	300	0.1	2.5	23.8	0.59	0.48	0.68
10	Batch	0.05	300	0.1	5	26.8	0.66	0.53	0.66
11	Batch	0.05	300	0.1	7.5	28.9	0.72	0.58	0.64
12	Fed-batch	0.05	300	0.1	10	39.7	0.67	0.72	0.69

Until now, an optimal initial concentration of biomass in the production process of succinic acid by *A. succinogenes* has not been determined in the literature, although a couple of studies have been carried out in which an attempt has been made to determine the optimum inoculum size, arriving at certainly different conclusions. On the one hand, Wan et al [43] observed that an inoculum size of 10% compared to 2% or 5% led to higher yields of succinic acid, which can be justified by relating a greater amount of cell density with reduced latency time. However, Anwar et al. [44] studied the effect of the inoculum size in a succinic acid production process through simultaneous saccharification and fermentation, concluding that increasing the inoculum size from 5% to 15% reduced the final concentration of succinic acid generated by 50%. The latter authors attributed this reduction in yield to the strong competition for nutrients that occurs in the culture broth when cell density is very high.

Considering these observations and the results of this work, it seems that an initial biomass concentration of an intermediate value is required that, on the one hand, is sufficient to avoid long latency times, but, on the other hand, does not lead to too rapid consumption of the carbon and nitrogen source. Other authors have also observed, in studies

carried out in different fermentation processes, that excessive initial amounts of biomass also lead to inhibitions by the product and accumulation of metabolites, which considerably impair the performance of the process [45,46].

However, these reflections do not seem to be sufficient to justify the increase in selectivity that occurs at higher initial biomass concentrations. This reduction in the number of by-products seems to be associated with possible variations in metabolism. To clarify this matter, it would be necessary to conduct metabolic analysis by carrying out fermentations with *A. succinogenes* under different inoculum size conditions. This was undertaken by Din et al. [47] with *Saccharomyces cerevisiae*, who observed large changes in glucose metabolism intermediates, amino acids, and metabolites related to the structure of the cell membrane by modifying the inoculum size.

In runs 1, 4, and 5, the CO_2 gas flow rate was increased from 0.1 to 1 L min^{-1}, values that include those that can be found in the literature on succinic acid production [10,11,13,23,26,37–42]. In this study, no significant variations in yield, productivity, or generation of by-products were observed in the range of flows studied. Taking into account the work of Xi et al. [17] and Zou et al. [18], who observed differences in the production of succinic acid working with mixtures of N_2 and CO_2 until reaching saturation of the latter gas, it is concluded that, as in the present work, fermentations with an excess of CO_2 do not favor the deviation of the metabolism towards the generation of succinic acid to the detriment of other metabolites. This means that most of the studies on succinic acid production by *A. succinogenes* published to date were carried out under conditions that involve higher economic costs and do not offer any additional advantage.

The increase in the agitation speed between 150 and 300 rpm, the range of values typically used in the literature [10,11,13,23,37–41,48,49], of runs 1, 6, 7, and 8, shows a considerable improvement in performance and productivity at high stirring values, reaching the best results at a stirring speed of 250 rpm. This operating condition is one of the factors with the greatest impact on the transfer of gases in liquid media, decreasing mixing time and improving mass and heat transfer rates [50,51]. Therefore, it can be deduced that the stirring values of 150 and 200 rpm are insufficient to achieve an adequate transfer of CO_2 in the culture broth. Taking this phenomenon into account, it could be deduced that the higher the stirring speed, the greater the generation of succinic acid; however, excessive shearing forces can lead to cell damage and, as a consequence, to the reduction in the process performance [52]. This seemed to happen in the run performed at 300 rpm, in which the effect of hydrodynamic stress appeared to be reflected.

In runs 1, 9, 10, and 11, the effect of the initial concentration of the nitrogen source in the culture medium was compared. As the YE concentration increased from 2.5 to 10 g L^{-1}, so did the succinic acid productivity. However, the yield of succinic acid with respect to the initial concentration of the carbon source reached its maximum in the fermentations carried out with 7.5 g L^{-1} of YE. However, it should be noted that Jiang et al. [33] achieved their maximum yield of succinic acid at around 20 g L^{-1} of YE, although they do not provide data on productivity or generation of by-products to be able to make a more detailed comparison with the present study. On the other hand, a tendency to improve selectivity was observed as the quantity of the nitrogen source decreased, which agrees with the conclusions of Ventrone et al [53], who observed that high C: N ratios lead to a reduction in by-product formation. In fact, in operations in the absence of a nitrogen source, with resting cells, Escanciano et al. [28] reduced the generation of by-products by 27.5% compared to the equivalent operation with cells in a state of growth, that is, in the presence of a nitrogen source.

The fed-batch run (run 12) reduced the number of by-products generated compared to the reference experiment, increasing the selectivity from 0.62 g g^{-1} (run 1) to 0.69 g g^{-1} (run 12). During their fed-batch production of succinic acid from citrus peel waste, Patsalou et al. [54] observed that the by-products were produced mainly in the first 24 h in a fermentation lasting more than 60 h, while the succinate continued to be generated throughout the entire process. In addition, these authors obtained a marked drop in

productivity, as occurred in the present work, which decreased from 0.83 g L^{-1} h^{-1} in the batch operation type to 0.72 g L^{-1} h^{-1} in the fed-batch fermentation. The loss in the production rate of succinic acid in this type of operation is a conclusion shared by more authors; for example, Kanchanasuta et al. [55] also saw a depletion in yield, a trend that was also observed in this work, although in a less pronounced way. Taking into account that succinic acid production appears to be favored in a non-growth steady state [56,57] and that nutrient depletion does not appear to be an obstacle to succinic acid production when there is already a high biomass concentration [28], it seems that the main problem caused by this type of operation is the excessive accumulation of metabolites, which can generate cell damage and strong inhibitions by product [7,13,37,58,59].

3.1. Development of a Simple Kinetic Model

For the development of the kinetic model, a reaction scheme was proposed based on the time course of the biomass, substrate (glucose), and fermentation products (succinic, acetic, and formic acids) of the reference experiment (run 1), whose evolution throughout over time is shown in Figure 1.A. It is observed that the biomass grows until reaching its maximum at 10 h of fermentation; however, both succinic acid and by-products continue to increase over time until the carbon source is exhausted around 33 h, indicating that production is not associated with growth. However, the rate of formation of acetic and formic acids slows down after approximately 20 h of fermentation, while the rate of production of succinic acid only suffers a slight reduction in the last hours of the process. In addition, although a greater amount of acetic acid is produced than formic acid, both compounds follow the same growth trend, which is why it was decided to combine both acids, as shown in Figure 1.B, with the aim of proposing a model with the metabolite "by-products" (BPs) that allows further reduction in the number of kinetic parameters and the development of a more useful model from the point of view of chemical engineering.

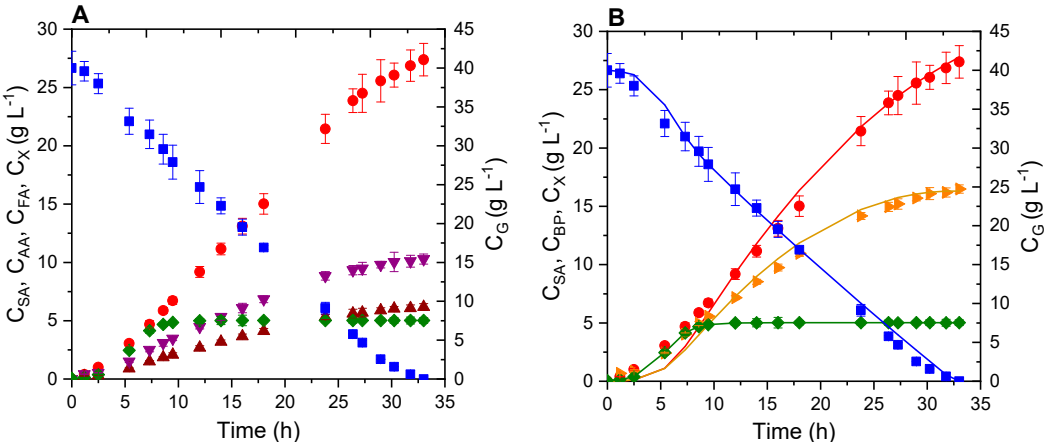

Figure 1. Evolution of the concentration of succinic acid (SA), acetic acid (AA), formic acid (FA), biomass (X), and glucose (G) over time in run 1 (reference). (**A**): representation of AA and FA concentrations separately. (**B**): combining of AA and FA concentrations into by-product (BP) concentration and kinetic model prediction. Data points: SA (●), G (■), AA (▼), FA (▲), X (♦), BP (►), model predictions shown as lines.

Based on these data, a very simple reaction scheme based only on Equations (1)–(3) is proposed. It is an unstructured non-segregated model, that is, the microorganism is considered as a single component, the biomass. This scheme is made up of a first reaction (r_1) for the consumption of glucose (S) for the generation of biomass, a second reaction (r_2) for the generation of succinic acid (P) and by-products (BPs), and a last reaction (r_3) for the independent generation of by-products. Their corresponding rates are shown in Equations (4)–(6), while the consumption and formation rates (R_j) of compounds 'j' are described in Equations (7)–(10). Therefore, the biomass has a growth rate based on the

logistic equation, whose kinetic parameters are the specific growth rate (μ) and the maximum biomass concentration (C_{Xm}). The generation of succinic acid and by-products is governed by potential equations independently of the growth of the microorganism in a proportional way to their kinetic constants K_{p1} and K_{p2}. In addition, together with these parameters, the formation and consumption rates are also defined by the macroscopic yields $Y_{S/X}$, $Y_{S/P1}$, $Y_{S/BP,}$ and $Y_{S/P2}$.

Reaction network

$$Y_{S/X}S \xrightarrow{r_1} X \quad (1)$$

$$Y_{S/P1}S \xrightarrow{r_2} P + Y_{S/BP} \cdot BP \quad (2)$$

$$Y_{S/P2}S \xrightarrow{r_3} BP \quad (3)$$

Reaction rates

$$r_1 = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_{Xm}}\right) \quad (4)$$

$$r_2 = k_{P1} \cdot C_S \cdot C_X \quad (5)$$

$$r_3 = k_{P2} \cdot C_S \cdot C_X \quad (6)$$

Production and consumption rates

$$R_S = \frac{dC_S}{dt} = -Y_{S/X} \cdot r_1 - Y_{S/P1} \cdot r_2 - Y_{S/P2} \cdot r_3 \quad (7)$$

$$R_P = \frac{dC_P}{dt} = r_2 \quad (8)$$

$$R_X = \frac{dC_X}{dt} = r_1 \quad (9)$$

$$R_{BP} = \frac{dC_{BP}}{dt} = Y_{S/BP} \cdot r_2 + r_3 \quad (10)$$

Applying this model, the calculation of its kinetic parameters (Table 2) was performed from the data of the reference experiment (run 1). The simulation of the evolution of the biomass, substrate, product, and by-products is shown in Figure 1.B, together with the experimental data, obtaining an excellent fit despite the simplicity of the model and the reduced number of parameters. In addition, Table 3 presents the statistical parameters that reflect the goodness of fit. A value of Fisher's F (F_{95}) of 41,242 was obtained, much higher than its tabulated value at 95% (where 8.55 is the value of F tabulated at that probability) and a percentage of explained variation very close to 100% (99.5%). In addition, very low values were obtained in those parameters that should be as close to zero as possible, with a sum of squared residuals (SSR) of 6.47 and a residual mean squared error (RMSE) of 0.67.

After verifying the validity of this model with the reference experiment, it was applied to all the other fermentations carried out under different operating conditions (runs 2–12), with the aims of verifying its robustness, studying the variation in the parameters, and knowing in greater depth the real impact of each of the operating conditions in the succinic acid production process. The estimated kinetic parameters for each of the experiments are shown in Table 2, while the statistical parameters are presented in Table 3.

Until now, the kinetic models of succinic acid production by *A. succinogenes* have been scarce and of the unstructured non-secreted type. In addition, they are usually

limited to the study of the rate of biomass formation, leaving aside the evolution of the metabolites present in the broth [12,60,61], although some authors such as Pateraki et al. [13] Lin et al. [37], and Vlysidis et al. [58] have also focused on the carbon source, as well as on the evolution of succinic acid and the generated by-products. Lin et al [37] proposed a biomass growth equation from glucose based on a combination of the Monod equation and the Luong equations for substrate and product inhibition. Vlysidis et al. [58] and Pateraki et al. [13] coincided in using the same combination of equations but used a Haldane–Andrews-type substrate inhibition term. Despite its low value, in all these works Pirt's maintenance coefficient is taken into account when estimating the consumption of the carbon source. In addition, they have in common that they resort to the Luedeking–Piret expression to predict the generation of succinic acid and by-products [7].

Although, on the one hand, they are very complete models that can provide a large amount of information about the fermentation process, on the other hand, the fact that they are precisely made up of long equations with a large number of parameters is impractical when, for example, carrying out an industrial scaling process, designing a control system, or carrying out a techno-economic analysis. In these circumstances, as demonstrated in this work, it is also possible to predict the evolution of the biomass and the same quantity of metabolites with a simpler system of equations based on the previous study of the relationship between the species present in the culture broth.

Table 2. Summary of kinetic parameters under different operational conditions.

Run	Type of operation	$C_{biomass}$ ($g \cdot L^{-1}$)	Agitation (rpm)	CO_2 Flow ($L \cdot min^{-1}$)	C_{YE} ($g \cdot L^{-1}$)	Cxm ± Error ($g_X \cdot L^{-1}$)	Kp_1 ± Error ($L \cdot g^{-1} \cdot h^{-1}$)	Kp_2 ± Error ($L \cdot g^{-1} \cdot h^{-1}$)	μ ± Error (h^{-1})	$Y_{S/P1}$ ± Error ($g \cdot g^{-1}$)	$Y_{S/P2}$ ± Error ($g \cdot g^{-1}$)	$Y_{S/BP}$ ± Error ($g \cdot g^{-1}$)	$Y_{S/X}$ ± Error ($g \cdot g^{-1}$)
1	Batch	0.05	300	0.1	10	5.02 ± 0.02	0.007 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	1.10 ± 0.09	1.65 ± 0.15
2	Batch	0.075	300	0.1	10	5.81 ± 0.03	0.009 ± 0.001	0.013 ± 0.001	0.85 ± 0.03	0.24 ± 0.02	2.20 ± 0.19	1.08 ± 0.07	1.63 ± 0.16
3	Batch	0.1	300	0.1	10	6.79 ± 0.08	0.004 ± 0.000	0.008 ± 0.001	0.85 ± 0.02	0.24 ± 0.02	2.25 ± 0.19	1.09 ± 0.08	1.63 ± 0.16
4	Batch	0.05	300	0.5	10	5.07 ± 0.03	0.008 ± 0.001	0.018 ± 0.001	0.85 ± 0.01	0.25 ± 0.02	2.27 ± 0.22	1.09 ± 0.09	1.66 ± 0.16
5	Batch	0.05	300	1	10	5.07 ± 0.03	0.008 ± 0.001	0.018 ± 0.001	0.85 ± 0.01	0.25 ± 0.02	2.27 ± 0.22	1.09 ± 0.09	1.66 ± 0.16
6	Batch	0.05	150	0.1	10	5.09 ± 0.09	0.004 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.26 ± 0.02	2.30 ± 0.21	1.10 ± 0.08	1.56 ± 0.13
7	Batch	0.05	200	0.1	10	5.05 ± 0.06	0.006 ± 0.001	0.018 ± 0.001	0.85 ± 0.06	0.26 ± 0.02	2.27 ± 0.18	1.04 ± 0.07	1.54 ± 0.11
8	Batch	0.05	250	0.1	10	5.00 ± 0.05	0.008 ± 0.001	0.018 ± 0.001	0.85 ± 0.05	0.24 ± 0.02	2.31 ± 0.18	1.07 ± 0.09	1.60 ± 0.13
9	Batch	0.05	300	0.1	2.5	2.40 ± 0.02	0.008 ± 0.001	0.022 ± 0.001	0.85 ± 0.05	0.24 ± 0.02	1.45 ± 0.10	0.86 ± 0.01	0.98 ± 0.02
10	Batch	0.05	300	0.1	5	5.01 ± 0.03	0.004 ± 0.001	0.011 ± 0.001	0.85 ± 0.03	0.25 ± 0.01	1.76 ± 0.11	0.95 ± 0.02	1.65 ± 0.02
11	Batch	0.05	300	0.1	7.5	5.08 ± 0.03	0.005 ± 0.001	0.014 ± 0.001	0.85 ± 0.04	0.24 ± 0.01	2.06 ± 0.17	1.00 ± 0.04	1.65 ± 0.04
12	Fed-batch cycle 1	0.05	300	0.1	10	5.02 ± 0.02	0.007 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	1.10 ± 0.09	1.65 ± 0.15
12	Fed-batch cycle 1	0.05	300	0.1	10	5.02 ± 0.02	0.007 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	0.59 ± 0.02	1.65 ± 0.15
12	Fed-batch cycle 1	0.05	300	0.1	10	5.02 ± 0.02	0.004 ± 0.001	0.005 ± 0.000	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	0.37 ± 0.01	1.65 ± 0.15

Table 3. Summary of statistical parameters under different operational conditions.

Run	Type of operation	$C_{biomass}$ (g·L^{-1})	Agitation (rpm)	CO_2 flow (L·min^{-1})	C_{YE} (g·L^{-1})	F_{95}	RMSE	SSR	VE %
1 REF.	Batch	0.05	300	0.1	10	41242	0.67	6.47	99.5
2	Batch	0.075	300	0.1	10	13660	1.09	11.26	98.2
3	Batch	0.1	300	0.1	10	40640	1.00	10.04	98.7
4	Batch	0.05	300	0.5	10	8457	1.14	12.17	98.5
5	Batch	0.05	300	1	10	8457	1.14	12.17	98.5
6	Batch	0.05	150	0.1	10	19384	0.92	9.01	99.0
7	Batch	0.05	200	0.1	10	19684	1.03	9.99	98.6
8	Batch	0.05	250	0.1	10	11751	1.07	11.70	98.5
9	Batch	0.05	300	0.1	2.5	5037	1.19	14.29	97.3
10	Batch	0.05	300	0.1	5	22441	1.10	11.57	98.9
11	Batch	0.05	300	0.1	7.5	17270	1.00	6.32	98.9
12	Fed-batch cycle 1	0.05	300	0.1	10	41242	0.67	6.47	99.5
12	Fed-batch cycle 1	0.05	300	0.1	10	13512	1.05	8.97	98.4
12	Fed-batch cycle 1	0.05	300	0.1	10	24175	0.95	6.09	99.6

3.2. Kinetic Study Based on the Initial Biomass Concentration

After applying the kinetic model to runs 1, 2, and 3, a good adjustment to the experimental data was achieved, as shown by the corresponding statistical parameters in Table 3. In these experiments, carried out at increasing initial biomass concentrations, it was observed that most of the estimated kinetic parameters did not suffer variations despite the modification of this operating condition (Table 2). However, three parameters of the model experienced considerable modifications and their variation is represented in Figure 2.

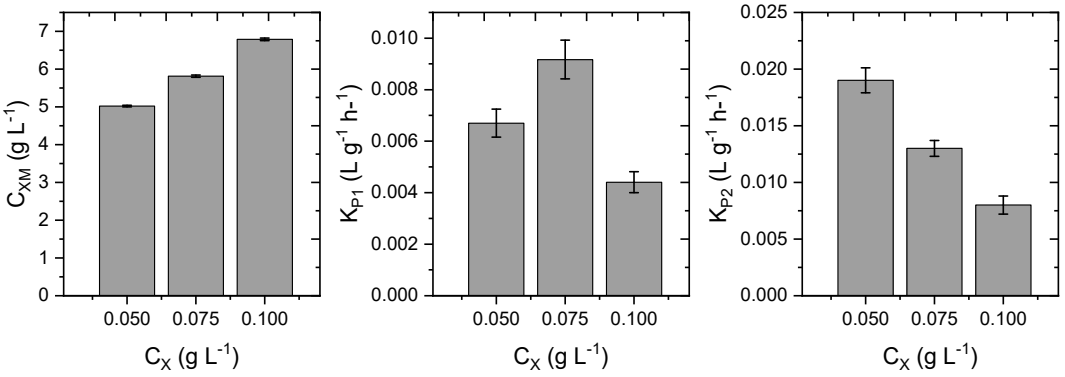

Figure 2. Kinetic parameters that are modified as a function of the initial biomass concentration.

On the one hand, the C_{xm} parameter shows that the increase in the initial biomass also leads to a higher maximum concentration of biomass in the culture broth once the stationary phase of its growth has been reached, with a practically linear trend, as expected. On the other hand, a drop in the kinetic constant of the by-product formation reaction (K_{P2}) is also observed, independent of that of succinic acid as the cell density increases in the broth, which agrees with the upward trend in selectivity previously discussed. Finally, the correlation between succinic acid productivity (Table 1) and the kinetic constant of the succinic acid formation equation (K_{P1}) is observed, reaching its maximum at an initial biomass concentration of 0.075 g L^{-1}.

3.3. Kinetic Study Based on the CO_2 Flow

As discussed in Section 3.1, despite modifying the CO_2 flow between values typically used in the literature [10,11,13,23,37–41,48,49], no variations were observed in the yield, productivity, or selectivity of the process (Table 1). The reason for this is that it seems that the excess of this gas does not propitiate the displacement of the metabolic route towards the formation of succinic acid. This deduction was confirmed with the application of the kinetic model to runs 1, 4, and 5, which enabled a simultaneous estimation of the three experiments without variation in the kinetic parameters (Table 2) and adequate goodness of fit (Table 3). Figure 3 shows the evolution of the experimental data of biomass, glucose, succinic acid, and by-products over time of runs 1, 4, and 5, together with the representation of the prediction of the evolution of their concentrations made on the whole.

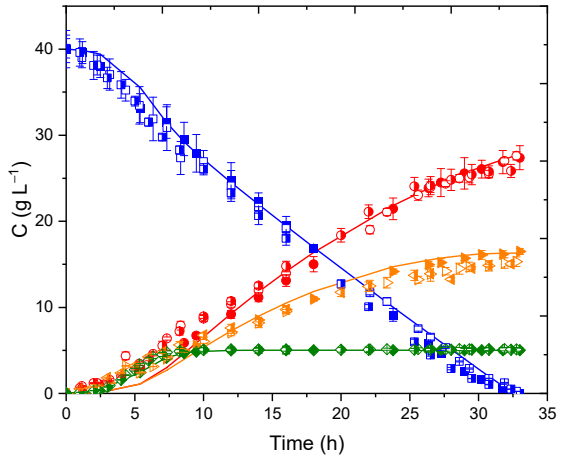

Figure 3. Kinetic model of the evolution of succinic acid (SA), by-products (BPs), biomass (X), and glucose concentrations (G) over time depending on CO_2 flow (0.1, 0.5, 1 L min^{-1}). Data points: SA (●), G (■), X (♦), BP (▶), 0.1 L min^{-1} close symbol, 0.5 L min^{-1} open symbol, 1 L min^{-1} half open symbol, model predictions shown as lines.

3.4. Kinetic Study Based on the Stirring Speed

Agitation is an operating condition whose increase favors the transfer of gas in the culture broth and the homogeneity of the compounds, in turn improving the productivity and yield of succinic acid (Table 1); in this case, the maximum reached was 250 rpm. However, above this speed, the cells seem to suffer damage, negatively affecting the development of the process. This behavior is reflected exactly in the parameters of the kinetic model, showing growth in the kinetic constant of the reaction for the formation of succinic acid (K_{P1}) until reaching a maximum at 250 rpm, and then a considerable reduction at 300 rpm, as shown in Figure 4.

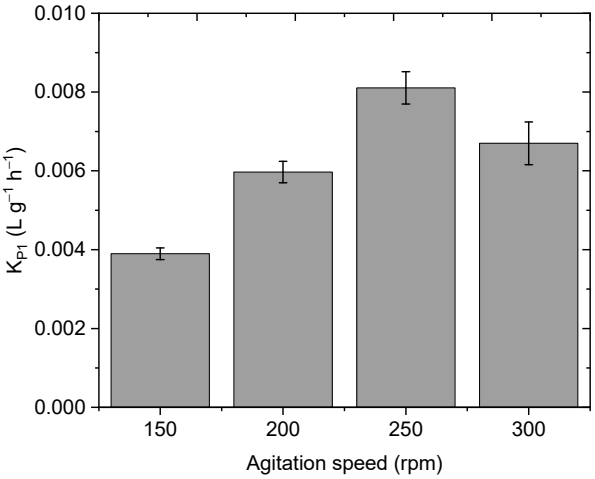

Figure 4. Kinetic parameters that are modified as a function of the stirring speed.

Despite the fact that, in this case, it was possible to observe an increase in productivity and possible cell damage, it should be noted that these conclusions differ from those of other authors such as Bevilaqua et al. [62], who did not observe significant variations in the performance of the process despite increasing the agitation up to 300 rpm, using the same microorganism as a biocatalyst although using hydrochloric hydrolysates of RH as the substrate instead of glucose. Gonzales et al [30] also studied the effect of stirring speed, performing experiments between 100 and 300 rpm. They only observed variations in the biomass concentration and determined that the optimum was to operate at low agitation speeds (100 rpm). These differences in the conclusions clearly open a door to the extension of the study of the influence of this operating condition, whose real impact seems yet to be determined.

3.5. Kinetic Study Based on the Yeast Extract Concentration

As discussed in Section 3.1, the concentration of yeast extract is a variable that has a great impact on the performance and productivity of the process, so that as the nitrogen source increases, higher productivity is achieved. However, the succinic yield peak was not reached at the maximum concentration studied (10 g L^{-1}), but at 7.5 g L^{-1}. Figure 5 shows that the kinetic estimations made in runs 1, 9, 10, and 11 led to the variation in six kinetic parameters. First, in the experiment at 2.5 g L^{-1} of YE (run 9), the values of the maximum biomass concentration (C_{xm}) and the macroscopic yield of biomass production ($Y_{S/X}$) were approximately half of those corresponding to all other fermentations carried out at higher concentrations of the nitrogen source (runs 1, 10, 11). Therefore, taking into account that there was not a great decrease in yield between run 9 and the rest of the experiments, it can be deduced that the amount of succinic acid and by-products generated per gram of biomass is much higher at 2.5 g L^{-1} of YE than in the experiments carried out at higher concentrations of YE, which results in an increase in the values of K_{P1} and K_{P2}.

It can also be observed that starting from 5 g L^{-1} of YE, the increase in the nitrogen source leads to the same increasing evolution of K_{P1} and K_{P2} as that of productivity. Despite this, an increase in the yield parameters related to the by-products ($Y_{S/BP}$, $Y_{S/P2}$) was observed as the initial concentration of YE increased, both in the simultaneous generation reaction of succinic acid and by-products (r_2), as well as in the isolated by-product generation reaction (r_3). This is consistent with the data presented in Table 1, that is, with the increase in selectivity with the decrease in the initial amount of YE.

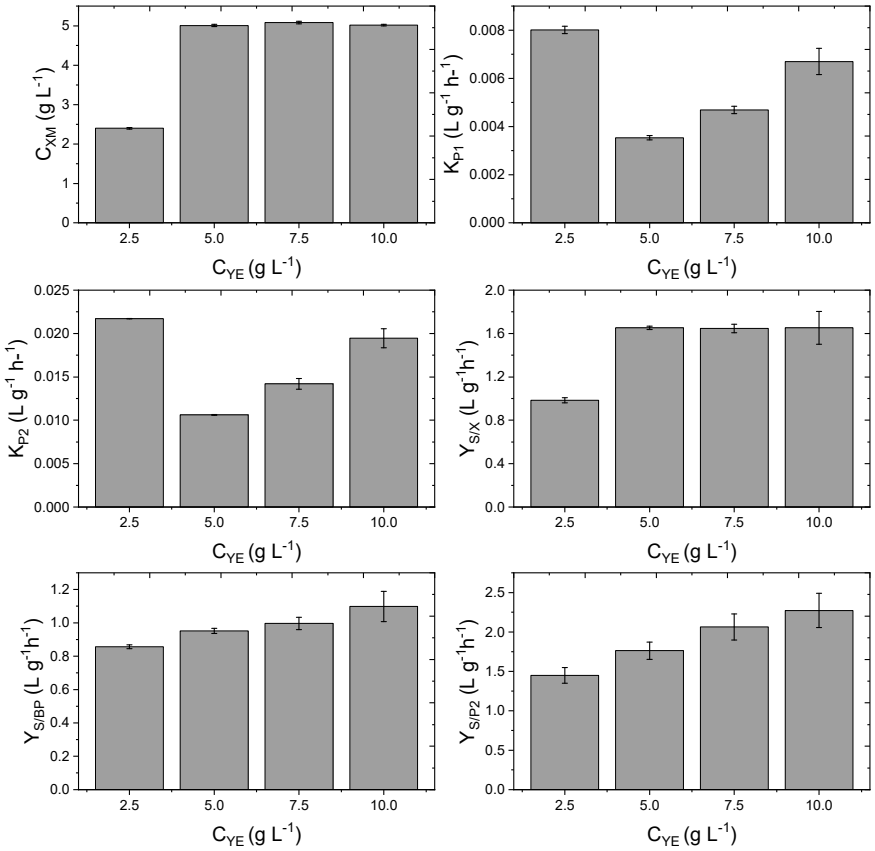

Figure 5. Kinetic parameters that were modified as a function of the yeast extract concentration.

3.6. Kinetic Estimation of the Stages of a Fed-Batch Type Operation

To check the robustness of the model for long fermentation times, a fed-batch fermentation (run 12) was performed. Kinetic estimates were made for each of the three stages, and simulations of the evolution of the biomass, substrate, product, and by-products over time were performed from maximum and minimum values of the kinetic parameters within the confidence interval. Figure 6 shows the experimental concentrations of biomass and metabolites over time, along with the prediction lines of the kinetic model and those simulated from the confidence interval. The yield values of succinic acid with respect to consumed glucose ($Y_{S/Gc}$) are also included, as well as the productivity and selectivity of succinic acid in the three stages. Figure 7 shows the kinetic parameters that underwent modifications throughout the three stages of the process.

It should be noted that, in the second stage, in line with the increase in selectivity with respect to the previous stage, there was a decrease in the yield parameter towards by-products (YS/BP) of reaction 2. Therefore, due to a greater use of glucose in succinic acid instead of by-products, it is logical that there is an increase in yield and productivity in this intermediate stage, without the need to alter any other kinetic parameter of the model.

Due to the increase in the selectivity of the third stage with respect to the previous stages, the macroscopic performance regarding by-products of reaction 2 ($Y_{S/BP}$) suffers a considerable reduction. However, the drop in succinic acid productivity is only reflected in the decrease in the kinetic constants of reactions 2 and 3 (K_{P1} and K_{P2}), parameters that decrease the reaction rate in a directly proportional manner. The simulation of the first cycle of run 12 could be carried out with the parameters estimated for run 1, since they share the same operating conditions.

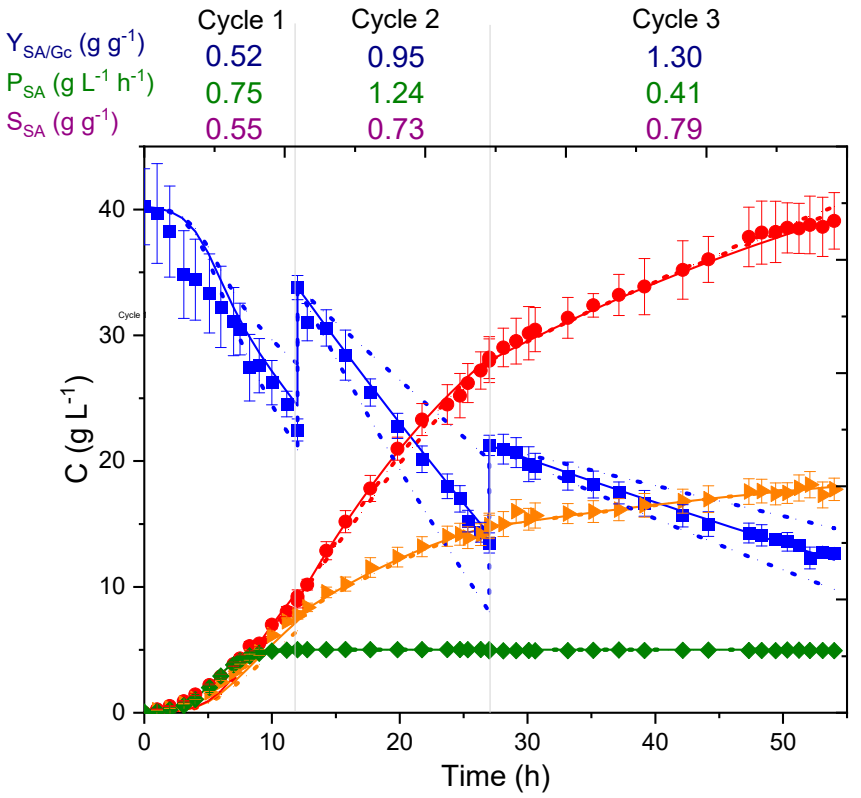

Figure 6. Evolution of the concentration of succinic acid (SA), by-products (BPs), biomass (X) and glucose (G) over time in run 11 (fed-batch). $Y_{SA/Gc}$: yield as a function of glucose consumed, P_{SA}: succinic acid productivity, S_{SA}: selectivity. Data points: SA (●), G (■), X (♦), BP (►), model estimations and simulations shown as lines.

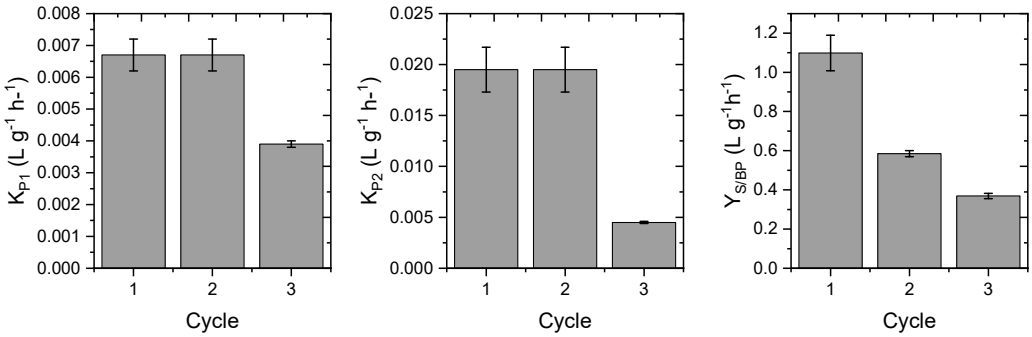

Figure 7. Kinetic parameters that were modified as a function of the fed-batch cycle.

4. Conclusions

In this work, an exhaustive study of critical variables in the bioproduction process of succinic acid by *A. succinogenes* was carried out to determine the effect of variables such as the initial concentration of biomass, the agitation speed, the concentration of yeast extract, and the CO_2 flow. A simple but robust kinetic model was developed which, unlike the models currently found in the literature, is capable of predicting the evolution of glucose, succinic acid, by-products, and biomass with few kinetic parameters. Its application to a reference run allowed verification of the goodness of fit, obtaining high values of F_{95} (41,242) and VE (99.5%), and values of RMSE and SSR close to zero. Subsequently, its validity was also demonstrated by estimating the evolution of metabolites in experiments in which the initial biomass concentration, the yeast extract concentration, agitation, and CO_2 flow were modified. Until now, these variables have not been used in the literature for the development of a succinic acid production kinetic model. Finally, the model was applied

to a fed-batch type operation, performing simulations based on the confidence intervals of the estimated parameters for each of the stages. In this way, it was possible to develop and validate a model, having significant robustness and simplicity, which is very useful from the point of view of chemical engineering for the scaling of the process, the design of a control system, or the performance of techno-economic analyses.

Author Contributions: Conceptualization, V.E.S. and M.L.; methodology, I.A.E.; software, I.A.E. and M.L.; validation, V.E.S., M.L. and Á.B.; formal analysis, V.E.S. and M.L.; investigation, I.A.E.; resources, V.E.S., M.L. and Á.B.; data curation, I.A.E. and V.E.S.; writing—original draft preparation, I.A.E.; writing—review and editing, V.E.S., M.L. and Á.B.; supervision, V.E.S. and M.L.; project administration, M.L. and Á.B.; funding acquisition, V.E.S., M.L. and Á.B. All authors have read and agreed to the published version of the manuscript.

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8.4 PUBLICACIÓN 4

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Título: Study on the operational modes using both growing and resting cells for the succinic acid production from xylose. Kinetic modelling

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Resumen:

El ácido succínico (SA) es uno de los principales productos químicos de plataforma obtenidos a partir de biomasa C4 que se pueden producir biológicamente. Hasta ahora, la mayoría de los productos de biorrefinería se han generar a partir celulosa y lignina, mientras que la hemicelulosa ha sido desatendida, siendo, sin embargo, la segunda fracción de polisacáridos más abundante en las lignocelulosas. En este artículo, presentamos resultados clave sobre diferentes alternativas operativas (*batch, fed-batch* y *repeated batch*) para mejorar la producción de SA empleando xilosa, el monosacárido más abundante en las hemicelulosas, utilizando células en crecimiento y en *resting* de *Actinobacillus succinogenes*. En primer lugar, se propuso un medio de crecimiento sintético económico y se empleó con éxito para la producción de SA. La reutilización de biocatalizadores demostró que el bioproceso se puede llevar a cabo con éxito en los modos de *repeated batch* y *fed-batch*. El mejor modo de operación con células en crecimiento es *repeated batch*: la concentración final de SA alcanzada es de 27,7 g L^{-1} y la productividad es de 0,50 g L^{-1} h^{-1}. En contraste, *fed-batch* fue el modo más conveniente con biocatalizador de células en *resting*, alcanzando una concentración y productividad final de 22,9 g L^{-1} y 0,53 g L^{-1} h^{-1}, respectivamente. Además, la formación de subproductos se reduce significativamente cuando se emplean células en reposo. Con ambos biocatalizadores, se desarrolla un modelo cinético no estructurado y no segregado capaz de simular el crecimiento celular, el consumo de xilosa, la producción de SA y la generación de subproductos, con una estimación exitosa de parámetros cinéticos respaldada por criterios estadísticos.

Study on the operational modes using both growing and resting cells for the succinic acid production from xylose. Kinetic modelling

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Production of succinic acid from xylose: a study on the operational modes using both growing and resting cells. Kinetic Modelling

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KEYWORDS: Succinic acid, xylose, *Actinobacillus succinogenes*, resting cells, kinetic model

ABSTRACT

Succinic acid (SA) is one of the main C4 biomass-based platform chemicals that can be obtained biologically. Till now, most biorefinery products have been developed on cellulose and lignin,

while hemicellulose has been widely neglected, being, however, the second most abundant polysaccharide fraction in lignocelluloses. In this paper, we present key results concerning different operational alternatives (batch, repeated batch and fed batch) to enhance SA production employing xylose, the most abundant monosaccharide in hemicelluloses, using *Actinobacillus succinogenes* growing and resting cells. First, a cost-effective synthetic growth medium was proposed and successfully employed for SA production. The biocatalysts reutilization showed that the bioprocess can be carried out successfully in repeated batch and fed-batch modes. The best mode with growing cells is repeated batch: final SA achieved titer is 27.7 $g \cdot L^{-1}$ and productivity is 0.50 $g \cdot L^{-1} \cdot h^{-1}$. In contrast, fed-batch was the most convenient mode with resting cell biocatalyst, reaching a final concentration and productivity 22.9 $g \cdot L^{-1}$ and 0.53 $g \cdot L^{-1} \cdot h^{-1}$, respectively. In addition, by-product formation is significantly reduced when employing resting cells. With both biocatalysts, a non-structured non-segregated kinetic model is developed able to simulate cell growth, xylose consumption, SA production and by-product generation, with successful kinetic parameter estimation supported by statistical criteria.

INTRODUCTION

Currently one of the main challenges in the fight against Climate Change is the search for renewable raw materials that replace fossil resources to generate energy, chemicals and materials. As a consequence, there is an urgent need of platform chemicals obtained through sustainable bioprocesses as alternatives to processes and products obtained via conventional refineries based on petroleum [1,2]. Most promising processes and products are being developed in second-generation biorefineries based on lignocellulosic biomass (LCB). LCB is especially attractive since it is the most abundant (~200 billion tons) and sustainable group of raw materials available worldwide and do not interfere in any food chain [3]. LCB is composed, in average, of cellulose (40-50%), hemicellulose (25-30%) and lignin (15-20%), to name the major components. Depolymerized hemicellulose is composed of 90% xylose, being this monosaccharide the second most abundant sugar available in LCB after glucose, a constituent of cellulose [4]. However, the number of wild-type microorganisms that can metabolize xylose as carbon source is very limited, so the hemicellulosic fraction of the waste is usually disposed of.

One of the most promising microorganisms to convert xylose into SA is a bacterium isolated from the rumen of cattle, *Actinobacillus succinogenes*. This microorganism transforms xylose into glyceraldehyde 3-phosphate through the pentose phosphate pathway. Subsequently, the bacterium

incorporates the last product of glycolysis, phosphoenolpyruvate, into the tricarboxylic acid cycle where, under anaerobic conditions and thanks to CO_2 insufflation, it is able to produce high amount of succinic acid (SA) [5,6].

According to US Department of Energy (US DOE), SA is one of the main chemical platforms due to its potential to produce a great diversity of chemicals traditionally produced from fossil sources [7]. This organic acid is widely used in the production of polybutylene succinate, polyester, polyols, in the food and pharmaceutical industries and to produce resins, coatings, pigments and biodegradable polymers [8].

In the recent years, the lack of sustainability of the traditional petrochemical production of SA has led to a considerable increase in its production volume through fermentation processes. In 2015 the market price of this acid produced by the biological route was 2.86 USD·kg^{-1}, while that obtained by the traditional route was 2.50 USD·kg^{-1} [9]. However, due to the great interest in biotechnological processes to SA, currently some economically competitive processes have already been developed and even implemented at an industrial level, producing SA with a market price between 2.00 and 2.50 USD·kg^{-1} [10]. With a 20% compound annual growth rate (CAGR), the bio-derived SA market is expected to reach 900 USD million by 2026, much higher than the

175.7 USD million value in 2017 [11]. The three main factors to consider in the implementation of a successful SA bioprocess at industrial scale are: (i) availability of raw material, (ii) SA adequate productivity and titer, and (iii) economically viable isolation and purification steps [12].

Ferone et al. (2017) [13] studied the production of succinic acid in batch operational mode by A. succinogenes employing a synthetic mixture of sugars representative of a lignocellulosic hydrolysis, obtaining a concentration of SA (27 $g \cdot L^{-1}$) higher than the ones obtained by combination of fermentations of each single sugar, as well as a better selectivity to SA. Using the same microorganism, Bukhari et al. (2020) [14] used oil palm trunk as carbon source after its hydrolysis with oxalic, formic, and acetic acid. Carrying out the bioprocess to SA with the hydrolyzed waste, they obtained a yield of 0.47 $g \cdot g^{-1}$ in batch bottle fermentation and a maximum concentration of succinic acid of 10.62 $g \cdot L^{-1}$. In order to enhance yield and productivity, some researchers have recently focused on optimizing the operational mode. Bradfield et al. (2016) [15] reached productivities of 3.4 $g \cdot L^{-1} \cdot h^{-1}$ and a maximum SA titer of 10.9 $g \cdot L^{-1}$ from xylose operating in continuous mode in a biofilm reactor. Jokodola et al. (2022) [16] opted for a fed-batch operational mode, producing 33.6 and 28.7 $g \cdot L^{-1}$ of succinate from hydrolysates of olive pits and sugarcane bagasse, respectively with same conversion yield (0.27 $g \cdot g^{-1}$). Considering that

downstream operations represent around 60% of the overall operating costs in SA production process, it is essential to reduce the by-products generation during fermentation in order to design a competitive process at an industrial level. For this, this research group carried out a previous study on the viability of succinic acid production employing a biocatalyst composed by A. succinogenes cells in a resting state [17]. Operating under nitrogen limited conditions, and formulating a medium composed exclusively of a carbon source and a buffer solution to maintain osmotic pressure, resting cells are maintained metabolically active even though cell growth is impeded. In this way, the metabolic pathways are active towards SA production and cellular maintenance but there is no bacterial growth. Concurrently, the number of by-products is also reduced dramatically [18–20]. In fact, Escanciano et al. (2022) [17] managed to reduce the quantity of by-products generated by 27.5% in the production of SA from xylose.

In the present work, we have investigated SA production using *A. succinogenes* in both growth and resting states, exploring different forms of operation through biocatalyst reuse by means of fed-batch and repeated batch. In addition, we intended to compare the effect on the production with cells in growth and resting state of the use of a cheaper culture medium than the one used in previous studies of the research group [17]. Finally, kinetic modeling of the bioprocess has been

developed to predict the evolution of biomass, xylose, succinic acid and by-products concentration throughout the fermentation time.

MATERIALS AND METHODS

Bacterial strain, adaptation, preinoculum and inoculum stages

The strain *Actinobacillus succinogenes* DSM 22257 was provided by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cultures GmbH. The bacterial strain was reactivated on one agar plate with Brain Heart Infusion (BHI) for two days. A single bacterial colony was inoculated in a bottle containing 60 mL of sterile Tryptic Soy Broth (TSB) [21–23]. The bottle was incubated for 1 day at 37 ˚C. For the long-term preservation, the culture was mixed with glycerol (1:1 $v \cdot v^{-1}$) and stored at -80 ˚C.

As it is described in a previous work [17], thawed bacteria were incubated at 37 °C for 24 h in anaerobic bottles with TSB medium. Afterwards, a two-step procedure was developed to adapt the biocatalyst to the carbon source. Broth from anaerobic bottles was employed as inoculum in a 5% ($v \cdot v^{-1}$) ratio by inoculating it in a second bottle containing production medium (PM) at 37˚C [24]. This production medium contained (in $g \cdot L^{-1}$): Yeast extract, 10; K_2HPO_4, 3; $MgCl_2.6H_2O$, 0.427; $CaCl_2$, 0.2; NaCl, 1; $NaHCO_3$, 10; xylose, 10. Using an inoculum of this second bottle, another

stage of adaptation in a third bottle was carried out, increasing the concentration of xylose and $NaHCO_3$ to 20 $g \cdot L^{-1}$ and the biomass initial inoculation volume from the previous step to 10% ($v \cdot v^{-1}$).

Succinic acid production employing growing cells biocatalyst

Batch, repeated batch and fed batch runs were carried out in a 2 L stirred tank bioreactor (STBR) BIOSTAT B-Plus (Sartorius AG, Germany) with a working volume of 1 L. Batch experiments have been carried out with PM medium. The initial amount of xylose was 20 $g \cdot L^{-1}$ in all runs. The pH was controlled at 6.8 (NaOH 5 M) at 37 °C and CO_2 was sparged at 0.1 vvm with a stirring speed of 300 rpm. The experiments were carried out starting from 0.05 $g \cdot L^{-1}$ of biomass in the exponential phase of growth, obtained in the final adaptation step in bottles.

Succinic acid production employing resting cells biocatalyst

To perform a fermentation employing cells in a resting state as biocatalyst, it is necessary to carry out previously a batch operational mode with growing cells as it was described in previous section to obtain a high amount of initial biomass. After 15 h of fermentation, suspended biomass was separated from the broth by centrifugation (9000 rpm, 5 min), washed with a K_2HPO_4 solution and transferred to a 1 L STBR with 0.5 L of working volume at 37 °C and 300 rpm. The production

media consisted in a buffered solution (K_2HPO_4 50 mM) of xylose 20 $g \cdot L^{-1}$. The pH was controlled at 6.8 (NaOH 5 M) and carbon dioxide was supplied at 0.1 vvm.

Succinic acid production operating fed-batch and repeated batch fermentations

Fed-batch and repeated batch runs were carried out with PM medium both with growing cells and resting cells biocatalysts, In the case of the fed-batch type operation, three stages were carried out, feeding a concentrated solution of xylose at the beginning of each one of them. In repeated batch fermentations, at the end of the first and second stages the suspended biomass was separated from the liquid broth by centrifugation and subsequently inoculated in the reactor of the next stage. The pH was controlled at 6.8 (NaOH 5 M), temperature at 37 °C, stirring speed at 300 rpm and CO_2 was sparged at 0.1 vvm.

Analytical methods

Biomass concentration was determined by measuring the optical density of broth samples at 600 nm using a spectrophotometer (Shimadzu UV-Vis spectrophotometer UV-1603).

Xylose and fermentation products (ethanol and succinic, lactic, acetic and formic acids) were analyzed through an Agilent Technologies 100 series equipment by high-performance liquid chromatography (HPLC) equipped with a refractive index detector (RID) at 55°C and a REZEX ROA-Monosaccharide H^+ (8%) column (300 x 7.8 mm, Phenomenex, USA) at 80°C. Acid water (H_2SO_4 5 mM) eluted at a flow of 0.5 $mL \cdot min^{-1}$.

Mathematical methods

The estimation of the kinetic parameters of the models has been carried out thanks to the computer software Aspen Custom Modeler v11 (AspenTech, USA), using an implicit Euler method to integrate the ODEs of the kinetic model coupled to a non -linear least-squares solver algorithm (NL2SOL) to obtain optimal values of the kinetic parameters.

To evaluate the goodness of fit, it should happen that Fisher's F-value (F) -Eq. (1)- has a higher value than its tabulated at 95% confidence, the sum of squared residuals (SSR) and the residual mean squared error (RMSE) are as close to zero as possible -Eq. (2)- and the variation explained (VE) is equal or near 100% -Eq. (3)-.

$$F = \frac{\sum_{i=1}^{N}\left(\frac{y_{i,\,calc}}{K}\right)^2}{\sum_{i=1}^{N}\left(\frac{SSR}{N-K}\right)} \quad (1)$$

$$RMSE = \sqrt{\frac{SSR}{N-K}} \quad (2)$$

$$VE(\%) = 100\left(1 - \frac{\sum_{l=1}^{L} SSQ_l}{\sum_{l=1}^{L} SSQ_{mean_l}}\right) \quad (3)$$

where K is the number of parameters, SSR is the squared sum of residues, N is the total number of experimental data, SSQ_l is the sum of the quadratic residues and SSQ_{meanl} is the squared sum of deviations between the experimental and the mean score respect to the calculated values.

In order to determine the influence of initial substrate concentration, consumed substrate, product distribution, and cell metabolism on SA production, the following parameters were defined:

Succinic acid yield: $\eta_{SA} = [SA]^{max}/[Xyl]_0$ (4)

Succinic acid macroscopic yield: $Y_{SA/Xyl.cons} = [SA]^{max}/[Xyl]_{cons}$ (5)

Specific succinic acid yield: $Y_{SA/X} = [SA]^{max}/[X]^{max}$ (6)

Selectivity on succinic acid: $S_{SA} = [SA]^{max}/([SA]^{max} + [BP]^{max})$ (7)

Succinic acid productivity: $P_{SA} = [SA]^{max}/time$ (8)

Specific succinic acid productivity: $P_{SA/X} = [SA]^{max}/([X]^{max} \cdot time)$ (9)

where $[SA]_{max}$ is the maximum concentration of succinic acid (SA), $[Xyl]_0$ is the initial concentration of xylose, $[Xyl]_{cons}$ is the concentration of consumed xylose, $[X]_{max}$ is the maximum concentration of biomass and $[BP]_{max}$ is the maximum concentration of by-products (BP).

RESULTS AND DISCUSSION

Influence of medium composition on succinic acid production employing growing cells and resting cells as biocatalyst

Growth medium composition as well as type and availability of carbon source play an important role on SA titre and productivity. Furthermore, the development of an effective biocatalyst is also a key factor that determines the viability of the bioprocess. Recently, *A. succinogenes* in resting state has been demonstrated as a promising biocatalyst, being a higher selectivity to the target acid the main advantage of resting cells when compared to growing cells [17]. In this section, the influence of medium composition on batch bioprocess is studied, comparing a synthetic growth medium (MP) to TSB, a complex growth medium employed for experiments conducted in previous published results.

Table 1 summarizes the obtained results in batch runs, in terms of the maximum product concentration achieved, and its selectivity, yields and productivities in each evaluated medium culture (MP and TSB) and biocatalyst state (growing and resting cells). Regarding product concentration, it is noticed that synthetic medium promotes SA production, whose final concentration in broth reaches the highest values when MP medium is employed (34% and 47% using growing and resting cells, respectively). As a consequence, in these conditions, high values

of SA yield with respect to initial carbon source are achieved (0.55 $g_{SA}\%g_{Xyl.0}^{-1}$ for growing state and 0.65 $g_{SA}\%g_{Xyl.0}^{-1}$ for resting state). However, in both cases, an increase of by-products production (acetic acid and formic acid) is appreciated, a fact that is reflected in a slight reduction of selectivity values. Nevertheless, the selectivity employing biocatalysts composing by resting cells is around 2-fold higher that the observed for growing cells.

Medium composition does not affect SA yield respect to final biocatalyst concentration employing growing cells. However, biocatalyst composed by resting cells cultivated in synthetic broth presents cell metabolism pathways more active to produce SA, as it is shown in the increase of this acid yield respect to biocatalyst concentration (2.91 $g_{SA}\%g_{X}^{-1}$) in comparison to the value when TSB is employed (2.03 $g_{SA}\%g_{X}^{-1}$). Similar tendency is observed for productivity (P_{SA}), whose meaning is closely linked to a macroscopic SA production rate. This acid productivity is boosted by MP medium employing resting cells biocatalyst, whereas is not improved in growing state. As the results showed, acid production from resting cells takes place 2.4-fold faster due to the change of the medium composition (with synthetic medium culture: 0.43 $g_{SA}\%L^{-1}\%h^{-1}$; when using complex medium culture: 0.18 $g_{SA}\%L^{-1}\%h^{-1}$).

Table 1. Concentrations, selectivity, yield and productivity on SA production with growing and resting cells and two different complex growth mediums in batch runs employing 20 $g \cdot L^{-1}$ of carbon source.

Biocatalyst	Growing cells		Resting cells	
Growth medium	TSB	MP	TSB	MP
C_{SA} ($g_{SA}\%L^{-1}$)	8.72	11.7	8.51	12.5
S_{SA} ($g_{SA}\%g_{SA+SP}^{-1}$)	0.64	0.55	0.75	0.68
η_{SA} ($g_{SA}\%g_{Xyl.0}^{-1}$)	0.44	0.55	0.43	0.65
$Y_{SA/Xyl.cons}$ ($g_{SA}\%g_{Xylcons}^{-1}$)	0.44	0.55	0.81	0.65
$Y_{SA/X}$ ($g_{SA}\%g_{X}^{-1}$)	2.80	2.74	2.03	2.91
P_{SA} ($g_{SA}\%L^{-1}\%h^{-1}$)	0.36	0.25	0.18	0.43
$P_{SA/X}$ ($g_{SA} \cdot g_{X}^{-1} \cdot h^{-1}$)	0.12	0.06	0.04	0.10
Reference	[17]	This work	[17]	This work

Based on these results, the employment of synthetic growth medium MP using *A. succinogenes* in growing and resting states enhances SA production in terms of final titre and yield respect to initial xylose concentration. In addition, productivity and yield respect to cell concentration is also improved for biocatalyst in resting cells. In short, synthetic medium MP is a promising alternative for the development of the bioprocess at industrial scale. Different operational modes using MP medium will be investigated in the following sections.

Figure 1 shows the evolution of substrate, product, by-product, and biomass concentrations in batch bioreactor employing MP medium. Substrate exhaustion is reached around 30 h for both biocatalysts. However, the experimental tendencies are different: whereas lag phase of growth leads to slower substrate consumption and product production rates at the beginning of the fermentation (Figure 1-A), a quick decrease on xylose concentration and, therefore, SA production is observed when a resting cells biocatalyst is employed (Figure 1-B). Even if the achieved target product concentration is similar in both cases, the global by-product concentration is reduced employing resting cells biocatalyst.

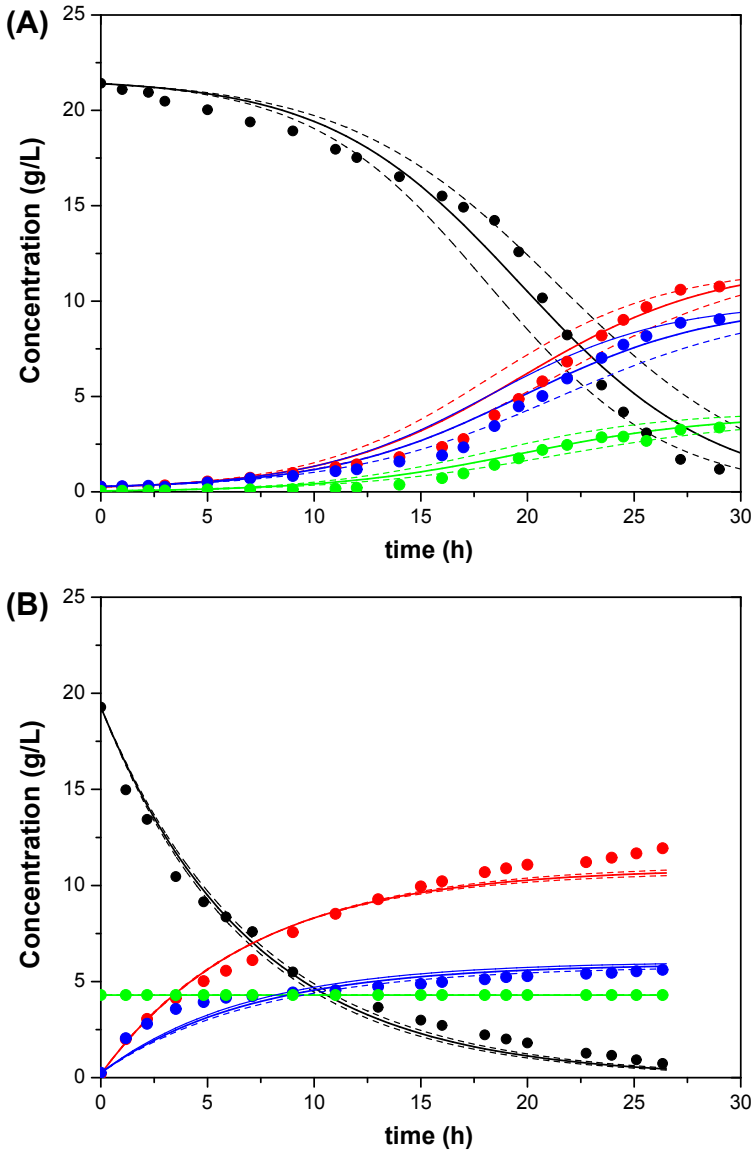

Figure 1. Evolution of substrate, product, by-products and biomass concentrations in batch bioreactor employing **(A)** growing cells biocatalyst, and **(B)** resting cells biocatalyst. ***Key:*** black: substrate, red: product, blue: by-product, green: biomass; dots: experimental data, solid lines: model prediction, dashed lines: error model prediction.

Succinic acid production in repeated batch bioreactor

In the present section, the possibility of reusing biocatalyst in three consecutive cycles was evaluated using a repeated batch bioreactor operation. Basically, repeated batch experiments explored the viability of separating biocatalyst at the end of the fermentation and, afterwards, harvested cells are employed as inoculum in the next cycle, as it was explained in section 2.4.

Each batch run was conducted employing around 20 $g \cdot L^{-1}$ of initial xylose concentration, which corresponds to the concentration range of carbon source presenting in vegetable raw materials [25]. When carbon substrate concentration reached 5 $g \cdot L^{-1}$, the experiment was stopped in order to avoid a metabolic stress to the bacterial cells. Afterwards, the biocatalyst was separated from broth by means of centrifugation and the recovered pellet was employed as biocatalyst in the next batch run. The main advantage of repeated batch in comparison to fed-batch operation is that repeated batch avoids inhibition due to accumulation of cytotoxic compounds in the broth, as it may occur along fed-batch.

Figure 2 shows the evolution of xylose, biomass, SA and by-products concentrations in repeated batch cycles evaluating the influence of the state of the biocatalyst: growing cells (Figure 2.A) and resting cells (Figure 2.B). While three consecutive cycles employing growing cells took less than 60 h, the use of the resting cells biocatalyst extended the fermentation time by 40 h. However, the

first cycle using biocatalyst composed by resting cells was much faster than the analogous experiment for growing cells, which is probably due to the greater biocatalyst concentration at the beginning and throughout the run. In the following steps, a deceleration of substrate consumption rate is observed when resting cells are reused. Biocatalyst concentration is progressively lost, because of the experimental cell recovery procedure between cycles. Nevertheless, the resting state biocatalyst was still active in second cycle, producing a similar amount of target product than in the first step. In the third cycle, the process suffered a significant deceleration, probably caused by the mechanical stress of the biocatalyst separation process and the scarcity of nutrients during the consecutive fermentation cycles.

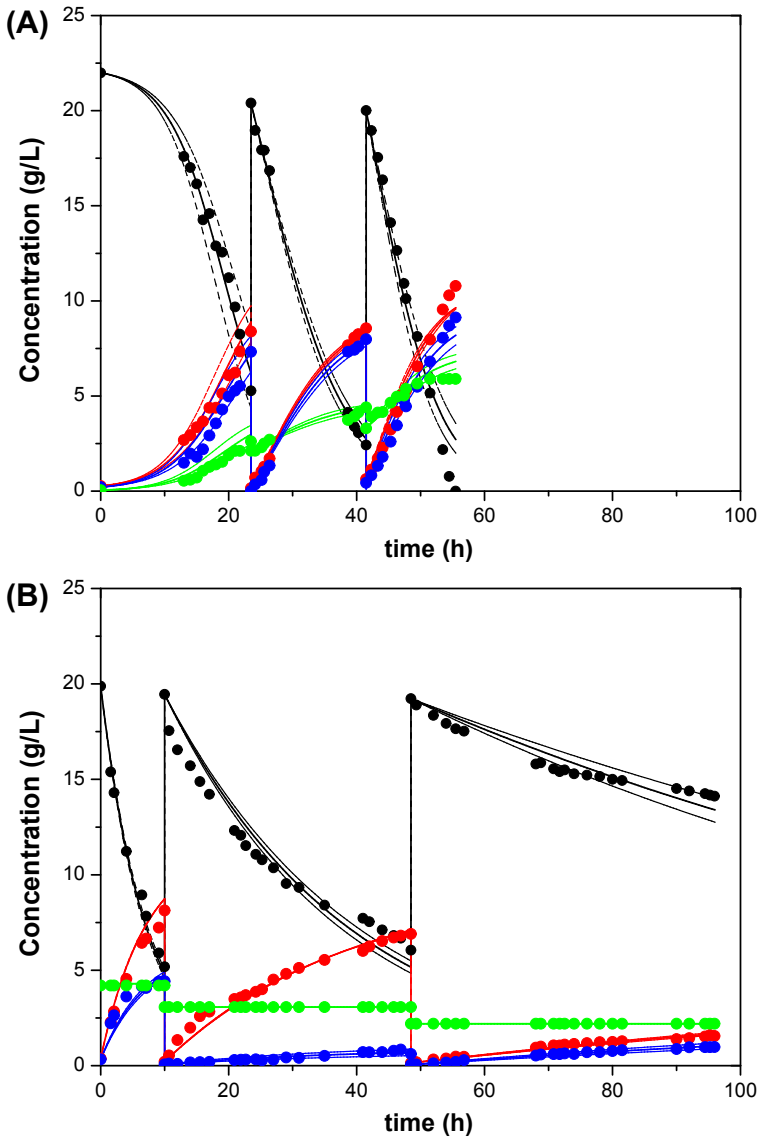

Figure 2. Evolution of substrate, product, by-product, and biomass concentrations in repeated batch bioreactor employing **(A)** growing cells biocatalyst, and **(B)** resting cells biocatalyst. ***Key:*** black: substrate, red: product, blue: by-product, green: biomass; dots: experimental data, solid lines: model prediction, dashed lines: error model prediction.

Regarding repeated batch working with the growing biocatalyst, it is to be noted that productivity is enhanced throughout the cycles, in terms of both substrate consumption and acid production as it is shown in Table 2. Although the initial biomass concentration at the beginning of each step is increased, the importance of the bacteria adaptation to carbon source plays also an important role in the bioprocess, as it has been previously demonstrated by Escanciano et al. [17]. In fact, the yield with respect to the xylose consumed and the productivity in the third stage are 72% and 56% higher than those corresponding to the first stage, respectively. Based on these results, the reutilization of the growing cell biocatalyst has been demonstrated, reaching 27.7 $g \cdot L^{-1}$ of overall SA concentration and a productivity of 0.50 $g \cdot L^{-1} \cdot h^{-1}$ (Table 2). Regarding specific SA productivity, cells in growing state are more productive per cycle than the resting cell biocatalyst. However, when the overall value is analyzed, it must be highlighted that there are no differences between both states (0.04 $g \cdot g^{-1} \cdot h^{-1}$).

Comparing both growing and resting biocatalyst behaviour in repeated batch operational mode, it should be pointed out that selectivity is strongly enhanced by resting state, the reutilization of growing cell biocatalyst leads to better overall SA production, in terms of titre, yield and productivity.

Table 2. Concentrations, selectivity, yields and productivity on SA production with growing and resting cells operating in repeated batch runs

Biocatalyst state	Growing cells				Resting cells			
Cycle	1	2	3	Overal l	1	2	3	Overal l
C_{SA} (g_{SA}%L^{-1})	8.39	8.56	10.8	27.7	8.13	6.90	1.55	16.6
S_{SA} (g_{SA}%g_{SA+BP}^{-1})	0.53	0.52	0.54	0.53	0.65	0.92	0.60	0.74
η_{SA} (g_{SA}%$g_{Xyl.0}^{-1}$)	0.38	0.42	0.54	0.51	0.41	0.35	0.08	0.28
$Y_{SA/Xyl.cons}$ (g_{SA}%$g_{Xyl.cons}^{-1}$)	0.50	0.48	0.54	0.51	0.55	0.51	0.30	0.50
$Y_{SA/X}$ (g_{SA}%g_X^{-1})	3.21	1.95	1.83	2.15	1.94	2.25	0.71	3.95
P_{SA} (g_{SA}%L^{-1}%h^{-1})	0.35	0.48	0.77	0.50	0.83	0.18	0.03	0.17
$P_{SA/X}$ (g_{SA}·g_X^{-1}·h^{-1})	0.13	0.11	0.13	0.04	0.20	0.06	0.01	0.04

Succinic acid production in fed-batch bioreactor

To avoid cell damage during separation processes between cycles, fed-batch operation was explored as alternative to extend operational time and, therefore, increase final SA titre and productivity. Three pulsed feeding were carried out (at the beginning and when substrate concentration was around 5 g·L^{-1}) to obtain similar conditions to repeated batch operation which allows the comparison of the results.

Figure 3 shows the evolution of xylose, biomass, SA and by-products concentrations in fed-batch operation employing different biocatalysts: growing cells (Figure 3.A) and resting cells

(Figure 3.B). Even if the total duration of both runs is similar, the allocated time for each pulse is different: substrate pulses are introduced at lower operation time values when employing resting cells. That is a consequence of a faster substrate consumption due to the higher biocatalyst concentration in comparison to the growing cells run. However, total substrate consumption was not reached in the last step in both cases. In fact, after 30 h of fermentation, only approximately half of the xylose fed in the third stage has been consumed. A significant deceleration of the process is observed in the last part of the experiments. This tendency may be caused by many factors: the exhaustion of essential nutrients that are not added in the pulsed fed, the aging of the cells or the presence of inhibition at high product concentration. It should be also stressed that, when employing resting cells as biocatalyst, SA production is utterly promoted over the by-products production, reaching a final SA concentration 3.6-fold higher that BP global concentration. Whereas, growing cells leads to a final SA concentration only 1.4-fold higher that BP global concentration.

Table 3 summarizes the obtained results in terms of final concentrations, selectivity, yields and productivity. It is notable that, while SA concentration and yields decreased between cycles in growing cells experiment, an increase was observed employing resting cells. Final product titre is

1.4-fold higher employing resting cells. In both cases, a shifting of the metabolic pathways towards SA production over BP production took place, as reflect the selectivity values. However, productivity was reduced dramatically. These results showed that the biocatalyst metabolism became slower thorough the cycles but more focused on the target product route.

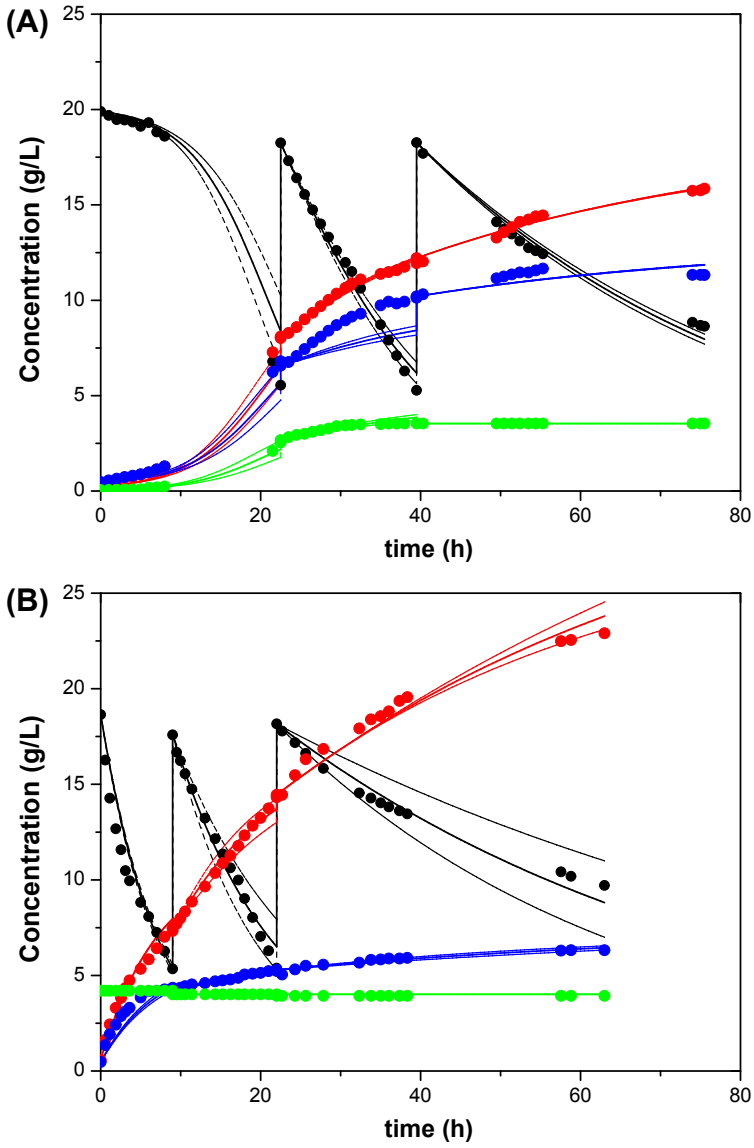

Figure 3. Evolution of substrate, product, by-product, and biomass concentrations in fed-batch bioreactor employing **(A)** growing cells biocatalyst, and **(B)** resting cells biocatalyst. ***Key:*** black: substrate, red: product, blue: by-product, green: biomass; dots: experimental data, solid lines: model prediction, dashed lines: error model prediction.

Table 3. Concentrations, selectivity, yields and productivity on SA production with growing and resting cells operating in fed-batch runs

Biocatalyst	**Growing cells**				**Resting cells**			
Cycle	1	2	3	Overal l	1	2	3	Overal l
C_{SA} (g_{SA}%L^{-1})	8.02	3.85	3.64	15.9	7.50	6.99	8.45	22.9
S_{SA} (g_{SA}%g_{SA+BP}^{-1})	0.54	0.52	0.76	0.57	0.63	0.87	0.89	0.78
η_{SA} (g_{SA}%$g_{Xyl.0}^{-1}$)	0.40	0.21	0.20	0.35	0.40	0.40	0.47	0.53
$Y_{SA/Xyl.cons}$ (g_{SA}%$g_{Xyl.cons}^{-1}$)	0.56	0.30	0.38	0.43	0.56	0.57	1.00	0.68
$Y_{SA/X}$ (g_{SA}%g_{X}^{-1})	3.15	1.09	1.03	4.48	1.79	1.74	2.15	5.46
P_{SA} (g_{SA}%L^{-1}%h^{-1})	0.36	0.23	0.10	0.21	0.83	0.54	0.21	0.36
$P_{SA/X}$ (g_{SA}·g_{X}^{-1} ·h^{-1})	0.14	0.07	0.03	0.06	0.20	0.13	0.05	0.09

Based on these results, the possibility of reusing biocatalyst by means of repeated batch and fed-batch operational modes has been successfully proved. Specifically, employing growing cells under repeated batch conditions and resting cells in fed-batch mode led to similar SA production (27.7 g·L^{-1} and 22.9 g·L^{-1}, respectively). In terms of productivity, production by growing cells in

repeated batch mode took place faster (0.50 $g \cdot L^{-1} \cdot h^{-1}$ and 22.9 $g \cdot L^{-1} \cdot h^{-1}$, respectively). Even more, the third cycle results did not show inhibition or aging cell phenomena. Thus, higher SA concentration might be reached adding new cycles in repeated batch operation with growing cells. On the contrary, metabolism of resting biocatalyst is shifted towards SA, as it was probed by yield with respect to consumed carbon source, yield referred to biomass concentration and selectivity values. These preliminary results established that the resting state is a promising biocatalyst condition; this work leaves the door open for a further optimization of the process (i.e. employing higher biocatalyst concentration, biocatalyst immobilization to facilitate product recovery and biocatalyst cyclic reuse –repeated batch- or use in continuous or fed-batch modes, etc.).

Table 4 summarizes published results regarding the production of SA mainly from xylose, using *A. succinogenes* as biocatalyst. Up to now, most of the studies have been performed in conventional submerged growth culture. The authors who carried out batch-type operations did not achieve yields greater than 55% [13] nor SA productivities higher than 0.36 $g \cdot L^{-1} \cdot h^{-1}$ [17]. However, substantially higher yields have been achieved with other types of operation, as in the case of Patsalou et al. [26], who managed to produce SA using citrus peel waste hydrolysate as renewable carbon source, reaching a yield of 0.73 $g \cdot g^{-1}$. So far, the fermentations carried out

operating in repeated batch mode have not been studied with xylose as a carbon source and have been carried out mostly with immobilized cells, which is why this work has focused in part on the production of SA from of xylose with repeated batch, achieving a yield of 51% and a productivity of 0.51 $g \cdot L^{-1} \cdot h^{-1}$.

The productions with A. *succinogenes* in a resting state were studied for the first time in a previous work of this research group [17], achieving a batch yield of 43 % while the productivity was 0.18 $g \cdot L^{-1} \cdot h^{-1}$; in this work, it has been possible to double the productivity and increase yield up to 53% thanks to the operation in fed-batch conditions.

Table 4. Summary of published studies aimed to SA production under different conditions (biocatalyst state, operational mode and carbon and nitrogen sources)

Biocatalyst state		Type of operation	Substrate	Nitrogen source	η_{SA} ($g_{SA}\%g_{substrate}^{-1}$)	P_{SA} ($g_{SA}\%L^{-1}\%h^{-1}$)	C_{SA} ($g_{SA}\%L^{-1}$)	Reference
Growing/Resting	Free/Immobilized							
Growing	Free	Batch	Xylose	Yeast extract	0.42	0.15	3.94	[27]
			Glucose, mannose, arabinose, xylose	Yeast extract	0.55	0.22	27.0	[13]
			Xylose	Yeast extract	0.44	0.36	8.7	[17]
			Xylose	**Yeast extract**	**0.55**	**0.25**	**11.7**	**This study**
		Fed-batch	Xylose	Yeast extract	0.27	0.51	36.7	[16]
			Xylose	**Yeast extract**	**0.35**	**0.21**	**15.9**	**This study**
			Citrus peel waste	Yeast extract	0.73	0.45	22.4	[26]
		Repeated batch	**Xylose**	**Yeast extract**	**0.51**	**0.50**	**27.7**	**This study**
	Immobilized	Repeated batch	Glucose	Yeast extract	0.39	1.32	33.6	[28]
			Glucose	Yeast extract	0.54	0.54	54.2	[29]
Resting	Free	Batch	Xylose	None	0.43	0.18	8.51	[17]

		Xylose	None	0.65	0.43	12.5	This study
	Fed-batch	Xylose	None	0.53	0.36	22.9	This study
	Repeated batch	Xylose	None	0.28	0.17	16.6	This study

Kinetic modelling of the bioprocess

Kinetic modelling allows for the simulation of bio/chemical processes and facilitates their implementation and operational control. To obtain a mathematical model able to fit kinetic data in this work, a very simple unstructured and non-segregated kinetic model is proposed which describes the observed experimental tendencies regarding xylose, biomass, SA, and by-products concentrations. The following considerations were taken into account:

- Laboratory-scale bioreactor was considered as completed mixed tank bioreactor.
- Acetic and formic acids concentrations were lumped or summed to obtain a global by-products concentration.
- Growth, SA production and BP generation depend proportionally on the availability of carbon source and the biocatalyst concentration.

Taking this information into consideration, a sole kinetic model (eq. 10-15) is developed to describe the behaviour of growing cells and resting cells biocatalysts for all the operational modes. Employing resting cells as biocatalyst, biomass concentration is constant and, therefore, yield biomass from product and biomass growth rate are zero.

- Reaction network $$Y_{S/P}\, S \xrightarrow{r} Y_{X/P}\, X + P + Y_{BP/P}\, BP \qquad (10)$$

- Reaction rate $$r = k_P \cdot [X] \cdot [S] \quad (11)$$

- Production and consumption rates

Substrate consumption rate $$R_S = \frac{d[S]}{dt} = -Y_{S/P} \cdot r \quad (12)$$

Product production rate $$R_P = \frac{d[P]}{dt} = r \quad (13)$$

Biomass growth rate $$R_X = \frac{d[X]}{dt} = Y_{X/P} \cdot r \quad (14)$$

By-products production rate $$R_{BP} = \frac{d[BP]}{dt} = Y_{BP/P} \cdot r \quad (15)$$

where $Y_{i/j}$ is the macroscopic yield of compound "i" with respect to compound "j" and k_P is the second-order kinetic constant in the SA production.

The fitting of the proposed kinetic equation to experimental data (substrate, biomass, product and by-products) for each cycle employing both biocatalysts was carried out to estimate the value of each kinetic parameter involving in the kinetic model. Model prediction fits very reasonably to all relevant data, as it is included in Figures 2 and 3 as solid lines. Then, the hypotheses assumed in the model are valid in the studied conditions, where no substrate or product inhibition phenomena were considered.

Tables 5 and 6 shows the estimated kinetic parameters, as well as the statistical parameters that provide the information on the goodness of fit. Fitting of experimental data regarding batch run

and first cycles of repeated batch and fed-batch runs were carried out together. Goodness-of-fit statistical parameters indicate a high value for Fisher's F, very much over the limiting value, and a low value for the RMSE and the SSR. Moreover, the experimental tendencies and the predicted from the model are quite similar, as the VE percentages higher than 90% showed.

The influence of biocatalyst state and reutilization cycles on the kinetic parameters values is shown in Figure 4. The second-order kinetic parameter k_P suffers a decreasing along the cycles of reutilization, independently of biocatalyst state and operational mode. These results point at a biocatalyst deactivation along the fermentation cycles. However, deactivation of growing cells under repeated batch takes place slower than for the other conditions. Regarding yields (substrate from product, by-product from product and biomass from product), smaller values indicate an effective SA production bioprocess, since less substrate and biomass are required for product generation with smaller side products generation. As it is reflected on yield tendencies, biocatalyst composed by resting cells is more specific for SA production, especially in the second and third uses.

Table 5. Kinetic and statistical parameter values calculated by fitting the kinetic model to experimental data of growing cells runs.

Cycle	Kinetic parameter	Operational mode		
		Batch	Repeated batch	Fed-Batch
1	k_P ($L \cdot g^{-1} \cdot h^{-1}$)		$(3.02 \pm 0.11) \cdot 10^{-2}$	
	$Y_{X/P}$ ($g \cdot g^{-1}$)		$(3.41 \pm 0.15) \cdot 10^{-1}$	
	$Y_{S/P}$ ($g \cdot g^{-1}$)		1.83 ± 0.03	
	$Y_{BP/P}$ ($g \cdot g^{-1}$)		$(8.21 \pm 0.20) \cdot 10^{-1}$	
2	k_P ($L \cdot g^{-1} \cdot h^{-1}$)	-	$(1.51 \pm 0.04) \cdot 10^{-2}$	$(6.50 \pm 0.19) \cdot 10^{-3}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	-	$(2.65 \pm 0.16) \cdot 10^{-1}$	$(2.88 \pm 0.25) \cdot 10^{-1}$
	$Y_{S/P}$ ($g \cdot g^{-1}$)	-	2.14 ± 0.04	2.94 ± 0.08
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	-	$(9.46 \pm 0.22) \cdot 10^{-1}$	$(9.39 \pm 0.03) \cdot 10^{-1}$
3	k_P ($L \cdot g^{-1} \cdot h^{-1}$)	-	$(1.38 \pm 0.07) \cdot 10^{-2}$	$(2.33 \pm 0.01) \cdot 10^{-3}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	-	$(3.89 \pm 0.42) \cdot 10^{-1}$	0.00
	$Y_{S/P}$ ($g \cdot g^{-1}$)	-	1.92 ± 0.08	2.77 ± 0.12
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	-	$(8.64 \pm 0.54) \cdot 10^{-1}$	$(4.45 \pm 0.05) \cdot 10^{-1}$
Cycle	Statistical parameter	Operational mode		
		Batch	Repeated batch	Fed-Batch
1	F		4715	
	RMSE		0.59	
	SSR		32.1	
	VE (%)		96.4	
2	F	-	8723	21562
	RMSE	-	0.24	0.25
	SSR	-	2.15	3.88
	VE (%)	-	99.7	98.8
3	F	-	1129	18932
	RMSE	-	0.80	0.32
	SSR	-	33.4	4.89
	VE (%)	-	96.0	96.1

Table 6. Kinetic and statistical parameter values calculated by fitting the kinetic model to experimental data of resting cells runs.

Cycle	Kinetic parameter	Operational mode		
		Batch	Repeated batch	Fed-Batch
1	k_P ($L \cdot g^{-1} \cdot h^{-1}$)		$(1.84 \pm 0.03) \cdot 10^{-2}$	
	$Y_{X/P}$ ($g \cdot g^{-1}$)		-	
	$Y_{S/P}$ ($g \cdot g^{-1}$)		1.80 ± 0.03	
	$Y_{BP/P}$ ($g \cdot g^{-1}$)		$(5.30 \pm 0.16) \cdot 10^{-1}$	
2	k_P ($L \cdot g^{-1} \cdot h^{-1}$)	-	$(5.36 \pm 0.14) \cdot 10^{-3}$	$(1.10 \pm 0.02) \cdot 10^{-2}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	-	-	-
	$Y_{S/P}$ ($g \cdot g^{-1}$)	-	2.08 ± 0.06	1.74 ± 0.04
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	-	$(9.17 \pm 2.49) \cdot 10^{-2}$	$(1.64 \pm 0.02) \cdot 10^{-1}$
3	k_P ($L \cdot g^{-1} \cdot h^{-1}$)	-	$(9.11 \pm 0.57) \cdot 10^{-4}$	$(4.43 \pm 0.10) \cdot 10^{-3}$
	$Y_{X/P}$ ($g \cdot g^{-1}$)	-	-	-
	$Y_{S/P}$ ($g \cdot g^{-1}$)	-	3.80 ± 0.25	$(9.99 \pm 0.29) \cdot 10^{-1}$
	$Y_{BP/P}$ ($g \cdot g^{-1}$)	-	$(6.39 \pm 0.74) \cdot 10^{-1}$	$(1.24 \pm 0.21) \cdot 10^{-1}$
Cycle	Statistical parameter	Operational mode		
		Batch	Repeated batch	Fed-Batch
1	F		3512	
	RMSE		0.61	
	SSR		57.1	
	VE (%)		96.8	
2	F	-	5090	19278
	RMSE	-	0.49	0.28
	SSR	-	20.4	4.54
	VE (%)	-	94.8	98.5
3	F	-	25099	20931
	RMSE	-	0.27	0.36
	SSR	-	6.09	7.15
	VE (%)	-	90.7	96.5

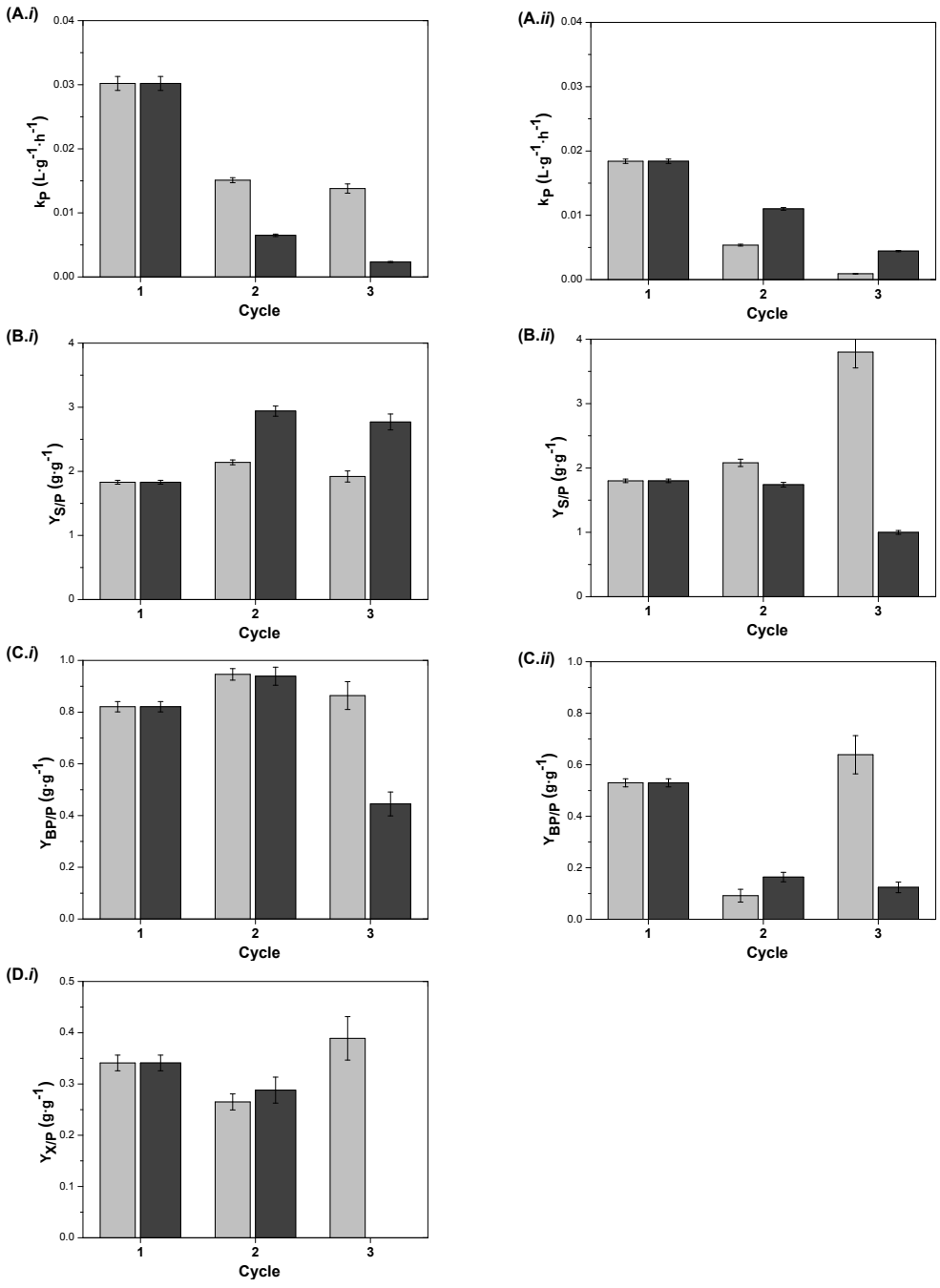

Figure 4. Influence of reusing (*i*) growing cells and (*ii*) resting cells biocatalyst under different operational mode on **(A)** second-order kinetic parameter of product formation, **(B)** pseudo-empirical stoichiometric coefficient substrate from product, **(C)** pseudo-empirical stoichiometric

coefficient by-product from product, and **(D)** pseudo-empirical stoichiometric coefficient biomass from product. ***Key:*** light grey: repeated batch, dark grey: fed batch.

CONCLUSIONS

In the present work, a bioprocess for SA production using a synthetic growth medium and xylose as carbon source has been demonstrated, which means an economic advantage in comparison to using a medium formulated based on complex carbon and nitrogen sources. Furthermore, the reutilization of biocatalyst composed by *A. succinogenes* cells under growing and resting states has been explored under diverse operation modes. Whereas the highest values of overall SA concentration and productivity were observed when growth state biocatalyst using repeated batch conditions, the highest yield and selectivity were reached by fed-batch operation with resting cells (whose nutrients availability was limited). In fact, in fed-batch at 55 h of fermentation, the yield was 59% higher with resting cells than with growing cells and one-third of by-products than with growing cells was also generated. Carrying out the bioprocess with resting cell biocatalyst presents several biological and technical advantages: (*i*) metabolic pathways to target product are promoted as indicated by the reduction in by-products generation, (*ii*) culture medium only contains phosphate and sugar, avoiding the use of expensive nitrogen sources, and (*iii*) SA is accumulated in the broth during the cycles, reaching a higher final concentration that produced employing growing cells biocatalyst. All these factors lead to reduce cost in the subsequent purification and

isolation operations. Thus, the possibility of producing SA with *A. succinogenes* in resting state becomes an innovative option with great potential and provides novel opportunities for the bioprocess intensification (e.g. biocatalyst immobilization).

Finally, a simple non-structured non-segregated kinetic model has been successfully developed to describe cell growth, SA production and by-product generation. The estimated kinetic parameters are supported by the observed experimental trends and give worthy information for a later bioprocess scale-up. In short, this research entails key progresses towards the incorporation of SA in biorefineries at industrial scale.

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M.L, V.E.S. Resources: M.L, V.E.S. Project administration: M.L, V.E.S, Funding acquisition: M.L, V.E.S.

Notes

The authors declare no competing financial interest.

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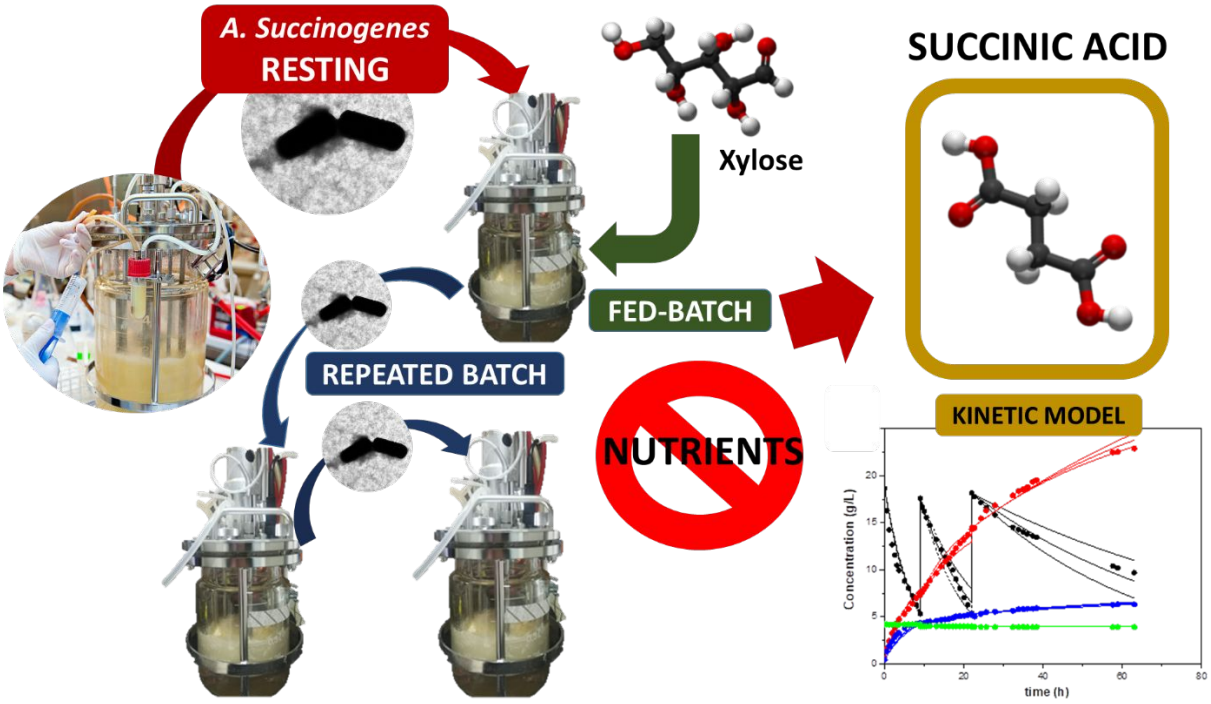
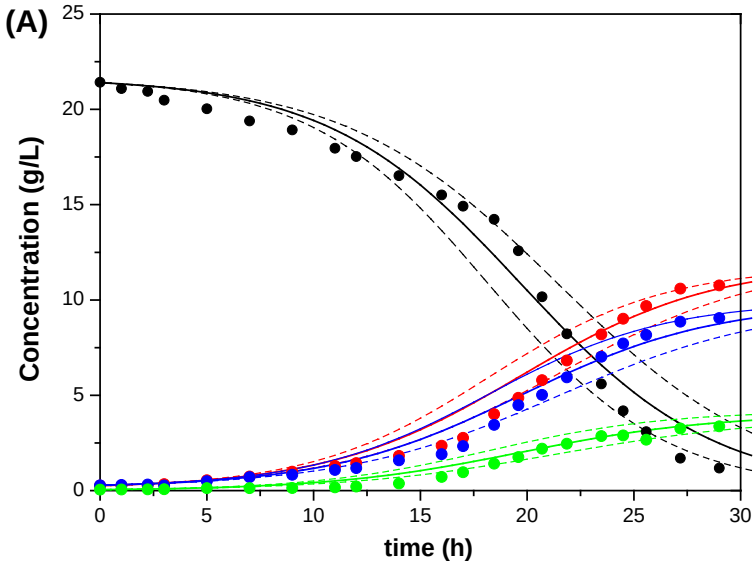
SYNOPSIS

Reduction of costs associated with the production of succinic acid with resting cells in fed-batch mode and kinetic modelling.

TOC/Abstract Graphic

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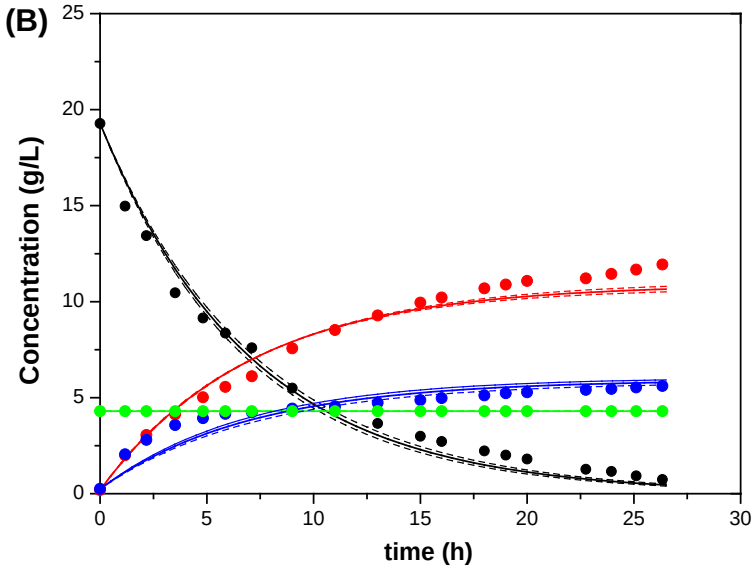
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10
15
20
25
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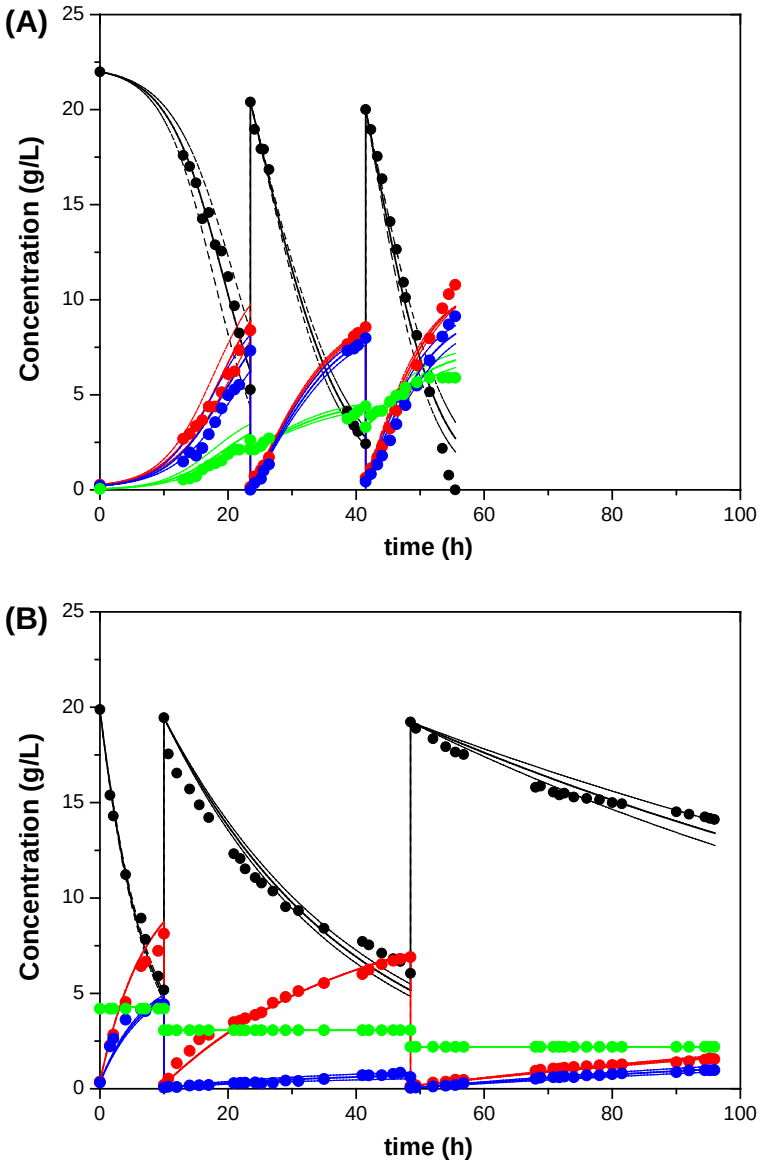
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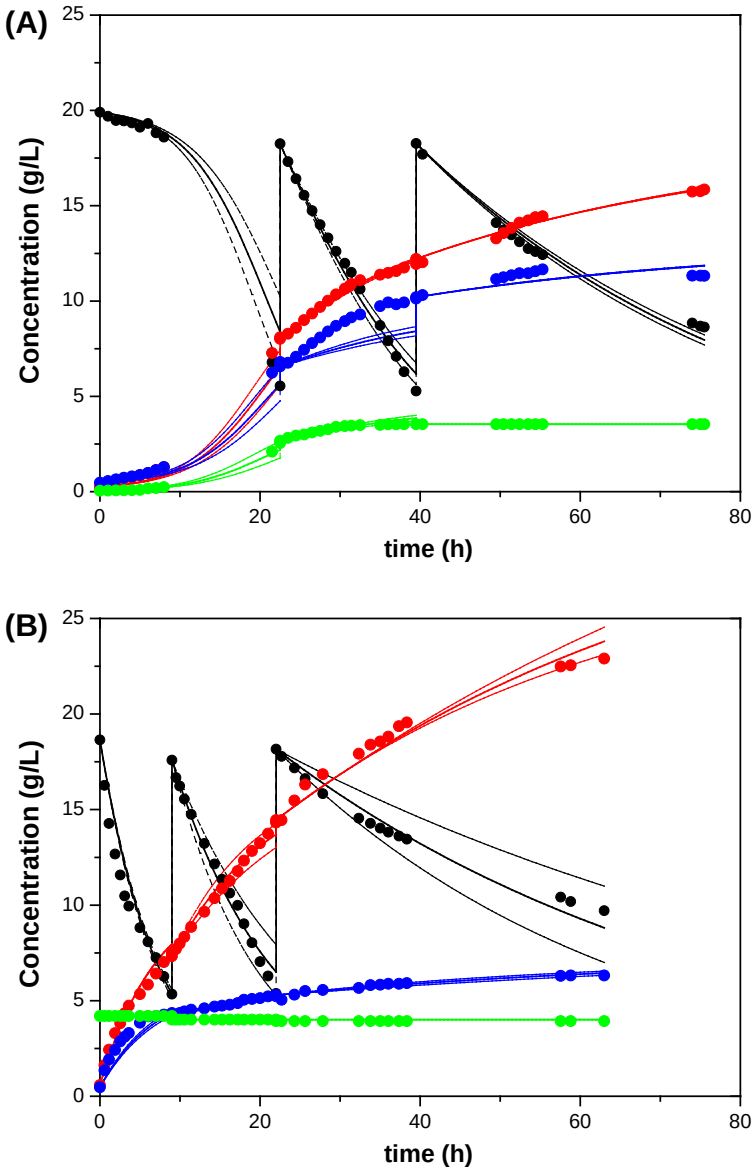
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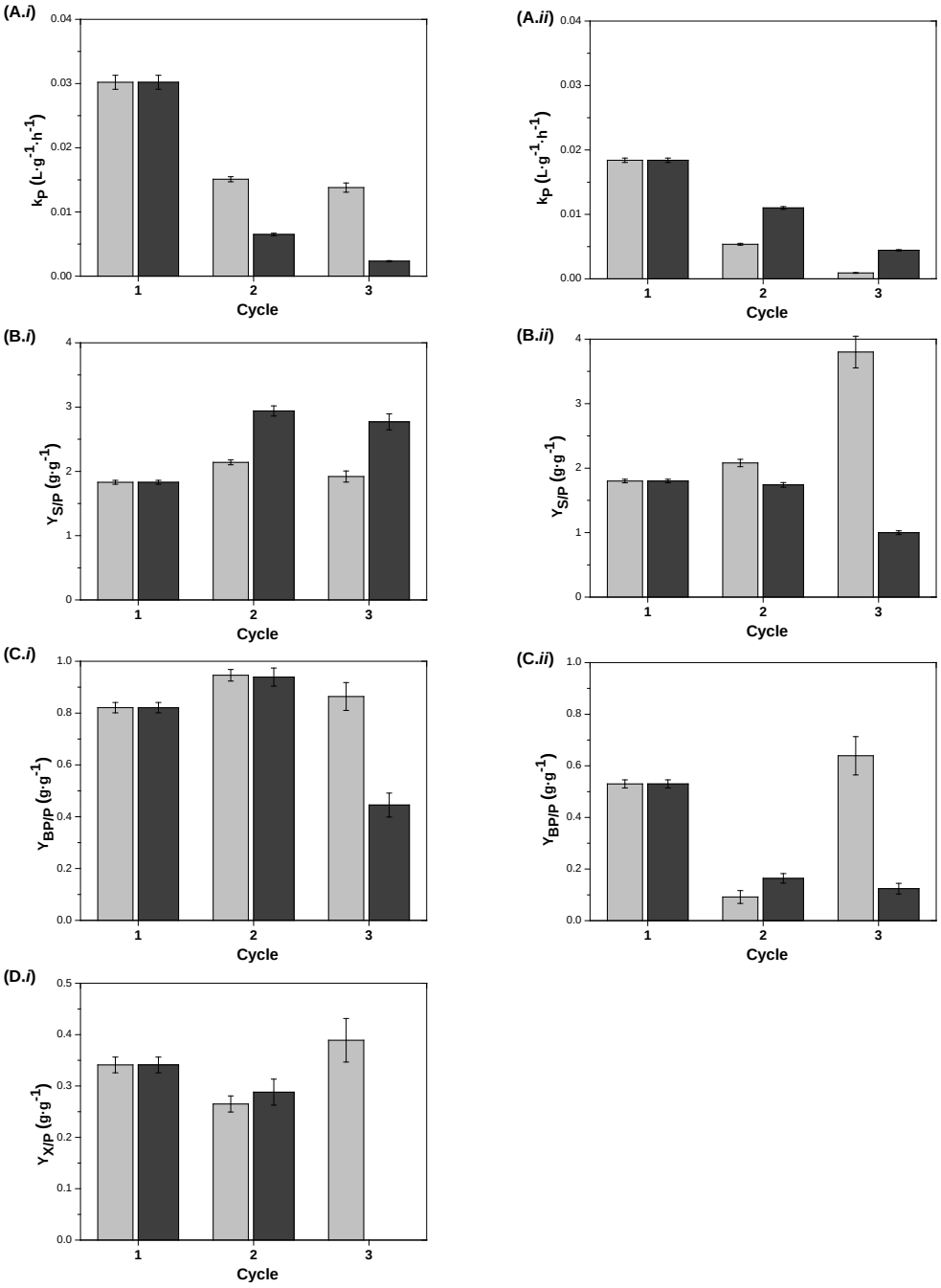
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Cycle
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0.02
0.01
0.00
1
2
3
Cycle
(B.i)
$Y_{S/P}$ (g·g^{-1})
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1
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1
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3
Cycle
(B.ii)
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3
2
1
0
1
2
3
Cycle
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2
3
Cycle
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2
3
Cycle
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0.1
0.0
1
2
3
Cycle

8.5 PUBLICACIÓN 5

Autores: Itziar A. Escanciano, Victoria E. Santos, Ángeles Blanco y Miguel Ladero

Título: Bioproduction of succinic acid from potato waste: process development and kinetic modeling

Revista: *Industrial Crops and Products* (en revisión)

Índice de Impacto (2021): 6,449

Categoría: Agronomía, Ingeniería Agrícola

Resumen:

El ácido succínico es un químico de plataforma clave para la implementación de las biorrefinerías y, por tanto, para el desarrollo de una bioeconomía sostenible. En este trabajo se llevó a cabo la producción de ácido succínico mediante *Actinobacillus succinogenes* a partir de residuos de patata. Primero, se desarrolló un modelo cinético simple a partir de experimentos realizados a diferentes concentraciones iniciales de glucosa, prediciendo con precisión la evolución del ácido succínico, el sustrato, los subproductos y la biomasa. Posteriormente, se realizaron experimentos en botella y reactor con un hidrolizado de desecho de patatas como fuente de carbono, comparando el rendimiento de la fermentación con experimentos equivalentes con glucosa pura. Con este hidrolizado se logró producir 32,2 g L^{-1} de ácido succínico con una productividad de 0,64 g L^{-1} h^{-1} y se mejoró el rendimiento en un 35% respecto a la fermentación con glucosa pura, obteniendo uno de los mejores resultados que se pueden encontrar en la bibliografía de bioproducción de ácido succínico a partir de residuos alimentarios (0,92 g g^{-1}). Finalmente, el modelo cinético se aplicó con éxito en la fermentación realizada a partir de residuos de patata, disponiendo así de una herramienta de gran valor para el desarrollo de análisis tecnoeconómicos, escalado del proceso o diseño de un sistema de control.

Industrial Crops & Products

Bioproduction of succinic acid from potato waste: process development and kinetic modeling

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
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Abstract:	Succinic acid is a key chemical platform for the implementation of biorefineries and, therefore, for the development of a sustainable bioeconomy. In this work, succinic acid production by Actinobacillus succinogenes from potato waste and glucose was carried out. First, a simple kinetic model was developed to accurately predict the evolution of succinic acid, substrate, by-products, and biomass based on bottle experiments performed at different initial glucose concentrations. Subsequently, experiments with an acid hydrolysate of potato waste as carbon source were carried out on bottle and reactor to compare the fermentation performance of the potato waste with the pure glucose. Using this hydrolysate, it was possible to produce 32.2 g L-1 of succinic acid with a productivity of 0.64 g L-1 h-1, improving the yield by 35% compared to fermentation with pure glucose, obtaining one of the highest bibliographic values for succinic acid bioproduction from food residues (0.92 g g-1). Finally, the proposed kinetic model was successfully applied to the fermentation data obtained from potato waste fermentation, being this model a highly valuable tool for future techno-economic analysis of the bioprocess.
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Bioproduction of succinic acid from potato waste: process development and kinetic modeling

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Abstract

Succinic acid is a key chemical platform for the implementation of biorefineries and, therefore, for the development of a sustainable bioeconomy. In this work, succinic acid production by *Actinobacillus succinogenes* from potato waste and glucose was carried out. First, a simple kinetic model was developed to accurately predict the evolution of succinic acid, substrate, by-products, and biomass based on bottle experiments performed at different initial glucose concentrations. Subsequently, experiments with an acid hydrolysate of potato waste as carbon source were carried out on bottle and reactor to compare the fermentation performance of the potato waste with the pure glucose. Using this hydrolysate, it was possible to produce 32.2 g L^{-1} of succinic acid with a productivity of 0.64 g L^{-1} h^{-1}, improving the yield by 35% compared to fermentation with pure glucose, obtaining one of the highest bibliographic values for succinic acid bioproduction from food residues (0.92 g g^{-1}). Finally, the proposed kinetic model was successfully applied to the fermentation data obtained from potato waste fermentation, being this model a highly valuable tool for future techno-economic analysis of the bioprocess.

Keywords: succinic acid; potato waste; hydrolysis; fermentation; kinetic model; CO_2

1. Introduction

The world population has not stopped growing since the middle of the last century, increasing from an estimated 2.5 billion people in 1950 to 8 billion in mid-November 2022 (Nations, n.d.). This growth rate of the world population and its consequences pose a great challenge for the Society. In the last 20 years, while the world population grew linearly by 20%, so did crop production, but at a rate of 43 % ((FAO), n.d.). A report from the Food and Agriculture Organization (FAO) concludes that around 1,300 million tons of food will be destroyed annually, for the cultivation of which it was necessary to use around 28 % of the available agricultural area ((FAO), n.d.; Sharma et al., 2021).

Thus, in 2015, the United Nations (UN) approved a set of global goals to eradicate poverty, protect the planet and ensure prosperity: the 17 Sustainable Development Goals (SDGs) (Leal Filho et al., 2022). To reach some of these goals the reuse of food waste is a must. In fact, SDG 12.3 calls for a 50% reduction in global per capita food waste at retail and consumer levels and a decrease in food loss along production and supply chains by 2030 ((FAO), n.d.). Furthermore, the amount of food wasted per year is projected to grow by a third by 2030, which involves 66 tons of food wasted per second (Esben et al., 2018). Therefore, considering these trends and the consequences of climate change, it is imperative to meet a sustainable economy through the valorization of wastes by a plethora of processes, including those of biotechnological nature. This means that circular economy concepts need to be further developed and biorefineries are part of the solution.

In the context of the biorefineries, succinic acid (SA) is one of the key compounds. It is considered one of the 12 main platform chemicals according to the United States Department of Energy (US DOE) (Dienst and Onderzoek, 2015). It has a wide variety of applications in the food and pharmaceutical industry. In addition, it is used for the generation of basic chemical products and there are great prospects for its use for the generation of biodegradable plastics in the bioeconomy era (Mancini et al., 2022; Oreoluwa Jokodola et al., 2022). As an intermediate compound in the tricarboxylic acid cycle, it can be produced from sugars through fermentation processes. Although many bacteria are capable of generating this acid it is only the main product in the metabolism of some of them, as in the case of *Actinobacillus succinogenes* in the presence of a CO_2 source from a wide variety of sugars (Escanciano et al., 2022a; Xu et al., 2022).

The global succinic acid market was valued at USD 160.8 million in 2022 and is expected to expand at a compound annual growth rate (CAGR) of 6.5% between 2022 and 2032, reaching a value of USD 301.4 million ("Succinic acid market," n.d.). The growing environmental awareness on the part of the governments of developed countries is strongly driving the increase in demand for bio-based succinic acid. The bio-succinic acid market is expected to account for a market share of 36.8% and revenue of USD 110.8 million by the end of 2032 ("Succinic acid market," n.d.). On the other hand, it should be noted that, in recent years, the cost of bio-based succinic acid has continued to decrease and even equalize the price of that from fossil resources. In 2015, the market price of this acid produced by the biological route was 2.86 $USD \cdot kg^{-1}$, while that obtained by the traditional petrochemical route was 2.50 $USD \cdot kg^{-1}$ (Dienst and Onderzoek, 2015). Currently, some economically competitive processes have been developed and even implemented at an industrial level, producing AS with a market price between USD 2.00 and 2.50 kg^{-1} (Mancini et al., 2022).

Based on the state of the art, an important challenge is the production of bio-succinic from wastes at a competitive yield. A waste with great potential for this use is potato waste. According to the Food and Agriculture Organization of the United Nations (FAO), the production of this tuber worldwide reached 370,436,581 tons in 2019 ((FAO), n.d.), being the fourth most important worldwide agricultural product after wheat, rice, and corn (Benkeblia, 2020). As potato processing, either domestic or industrial, generates a waste whose mass is 15-40% of potato weight, potato waste global yearly production is estimated to be 55-140 million tons (Ebrahimian et al., 2022). The main by-products of potato processing are: potato peels, discarded potatoes (old potatoes and low quality ones), potato leaves and starch processing wastewater, all of which are rich in carbohydrates, proteins and antioxidants (Torres and Domínguez, 2020).

This work showcases for the first time the production of SA from potato waste as a carbon source using *A. succinogenes* as biocatalyst. Herein, we compare the yield and productivity of the fermentation process in relation to its equivalent with pure commercial glucose, carrying out batch operations both in bottle and in bioreactor. Finally, we develop a kinetic model that allows predicting the evolution of biomass, substrate and products in an easy way. This mathematical model could serve as a tool for process scale-up, design of control systems and/or for performing techno-economic analyses.

2. Materials and methods

2.1. Raw materials and hydrolysis procedure

The raw material used in this study were discarded potato pieces kindly provided by ESPAFRIMA S.L (Getafe, Madrid). The potato pieces were washed, peeled and crushed until they were made into a puree. Subsequently, an acid hydrolysis was carried out (Gunnarsson et al., 2014; Huang et al., 2019; Tasić et al., 2009). A 0.5 L round bottom flask with a reflux condenser was used. The process temperature was controlled with a heating mantle. 150 mL of 1M HCl was poured into the flask and heated to 100 °C; then 150 g of mashed potato were added and 3 mL samples were taken every 10 minutes for two hours. Samples were neutralized with 5 M KOH and centrifuged at 9,000 rpm for 10 minutes. Finally, the concentration of sugars present in the supernatant was determined, as well as furfural and hydroxymethylfurfural (HMF).

2.2. Microorganism, culture medium and fermentation procedure

Actinobacillus succinogenes DSM 22257 was supplied by the German Collection of Microorganisms and Cell Cultures GmbH. The strain was maintained at -80 °C in Tryptic Soy Broth (TSB) /glycerol 50 % v v^{-1}. TSB composition was (in grams per liter): 17 Tryptone, 3 Soytone, 2.5 Glucose, 5 NaCl, 2.5 K_2HPO_4.

For its reactivation, the stored cells were injected into bottles with 60 mL of TSB. These bottles have been previously purged with N_2 for 2 minutes at a 1 L min^{-1} flow rate. For pH control and as a CO_2 source, $NaHCO_3$ was added in the same concentration as the carbon source. The cells were incubated at 37 °C and 200 rpm for 24 h. After adding 5% (v v^{-1}) of reactivated cells, the adaptation of the microorganism to the carbon source was carried out by means of successive growth cycles in bottles under the same conditions of the reactivation stage, but with increasing concentrations of sugar and using the production medium. The production medium was (in grams per liter): 3 K_2HPO_4, 0.43 $MgCl_2.6H_2O$, 0.2 $CaCl_2$, 1 NaCl, 10 yeast extract. Finally, for the growth of the inoculum, 5% (v v^{-1}) of adapted cells were injected into bottles prepared and incubated under the same conditions of the adaptation stage, with 40 g L^{-1} of glucose. When the inoculum was used in a fermentation process with potato hydrolysate, an adaptation stage to the hydrolysate in the bottle was added, increasing the hydrolysate/pure glucose ratio in successive steps until it completely replaced the commercial sugar.

For the comparative study of the fermentation in bottles with commercial glucose or from potato residues, incubations were carried out at 37 ºC and 200 rpm using the production medium and 40 g L^{-1} of pure glucose or potato hydrolysate in the amount enough to reach the same concentration. After measuring the cell concentration in the inoculum, a sufficient amount of biomass was injected to start fermentation from 0.05 g L^{-1} of biomass.

The runs in reactor were performed in a 2-L stirred tank BIOSTAT B-Plus (Sartorius AG, Germany) with a working volume of 1 L. The previously described production medium was used. The carbon source was 40 g L^{-1} of commercial glucose or potato hydrolysate in sufficient quantity to reach the same sugar concentration. Fermentation was carried out at 37 ºC, with a stirring speed of 300 rpm, pH 6.8 (controlled

by automatic addition of 5 M NaOH) and a CO_2 flow rate of 0.1 vvm. The initial biomass concentration was 0.05 g L^{-1}.

2.3. Analytical methods

A spectrophotometer (Shimadzu UV-Vis spectrophotometer UV-1603) was used to measure the cell optical density at 600 nm of the samples.

The concentration of sugars, furfurals and carboxylic acids was calculated from the measurements made by a refractive index detector (RID) at 55 ºC of a high-performance liquid chromatography (HPLC) equipment (Agilent Technologies 100 series). A REZEX ROA-Monosaccharide H^+ (8%) column (300 x 7.8 mm, Phenomenex, USA) working at 80 ºC was employed, pumping 0.5 mL min^{-1} of a 5 Mm H_2SO_4 solution as mobile phase.

2.4. Mathematical methods

Based on the evolution of the concentrations of glucose, succinic acid and by-products (acetic and formic acids), a kinetic model has been proposed whose parameters have been adjusted to the experimental data thanks to the computer software Aspen Custom Modeler v11 (AspenTech, USA). In order to integrate the differential equations and estimate the parameters applying the least square method, an implicit Euler method coupled to a non-linear least-square solver algorithm (NL2SOL) has been used.

The statistical parameters that have been studied to determine the goodness of fit are calculated using equations (1-3). Fisher's F-value (F) (Eq. (1)) should be higher than its tabulated value at 95% confidence to overcome the null hypothesis; over this threshold, the higher its value, the better. The Root Mean Square Error (RMSE) (Eq. (2)) is based on the variance computed with the experimental values and those values predicted by the kinetic model; when both values are identical in all conditions, variance and this parameter are zero, showing a perfect fit of the model to the experimental data. The variation explained (VE) measures the capacity of the kinetic model to explain the variation of the dependent variables with the independent variables (in this case, the process time); its best value is 100 %.

$$F = \frac{\sum_{i=1}^{N}\left(\frac{y_{i,calc}}{K}\right)^2}{\sum_{i=1}^{N}\left(\frac{SSR}{N-K}\right)} \quad (1)$$

$$RMSE = \sqrt{\frac{SSR}{N-K}} \quad (2)$$

$$VE(\%) = 100\left(1 - \frac{\sum_{l=1}^{L} SSQ_l}{\sum_{l=1}^{L} SSQ_{mean_l}}\right) \quad (3)$$

$y_{i,calc}$ are the estimated values, K is the number of kinetic parameters, SSR is the sum of variances or squared residues, N is the number of experimental data, SSQ_l is the sum of the quadratic residues and SSQ_{meanl} is the squared sum of deviations between the experimental and the mean score with respect to the calculated values [10].

To determine the impact of the carbon source on the fermentation process, the following parameters have been calculated: the yield of the product (P), succinic acid, with respect to the initial concentration of the substrate (S), which is glucose ($Y_{S.0}$ – Eq. (4)), the yield of the product with respect to substrate consumed ($Y_{S.cons}$ – Eq. (5)), the succinic acid productivity (Prod – Eq. (6)) and the succinic acid selectivity (Sel. – Eq. (7)).

$$Y_{S.0} = \frac{C_{P.max}}{C_{S.0}} \quad (4)$$

$$Y_{S,cons} = \frac{C_{P.max}}{C_{S.cons}} \quad (5)$$

$$Prod. = \frac{C_{P.max}}{time} \quad (6)$$

$$Sel. = \frac{C_{P.max}}{C_{P.max} + C_{BP,max}} \quad (7)$$

$C_{P,max}$ is the concentration of SA at the end of the fermentation, $C_{S,cons}$ is the difference between the glucose concentrations at the beginning and at the end of the fermentation and $C_{BP,max}$ is the concentration of the sum of the by-products (BP) (acetic and formic acids) at the final time of fermentation.

3. Results

3.1. Determination of the optimal hydrolysis time

During the acid hydrolysis of potato peels at 100 ºC, glucose (substrate, S) was produced from amylose and amylopectin at a high rate (1.8 g L^{-1} min^{-1}) till 40 minutes, when a certain and progressive decrease in temporal productivity is appreciated in Figure 1. The dehydration product of glucose, 5-hydroxymethylfurfural (HMF), increases from 20 minutes of reaction with an increasing productivity or production rate. A processing time of 60 minutes shows the maximum difference between the concentration of glucose, the target product, and HMF, while HMF concentration is still relatively low. Higher processing times only render a high to very high amount of HMF while glucose increment is limited. To reduce the deleterious action of HMF on the microorganism (Tan et al., 2021), a 60 minutes processing time was chosen as the hydrolysis time to obtain the glucose solution for SA bioproduction, maximizing the difference between the target substrate, glucose, and the undesired by-product.

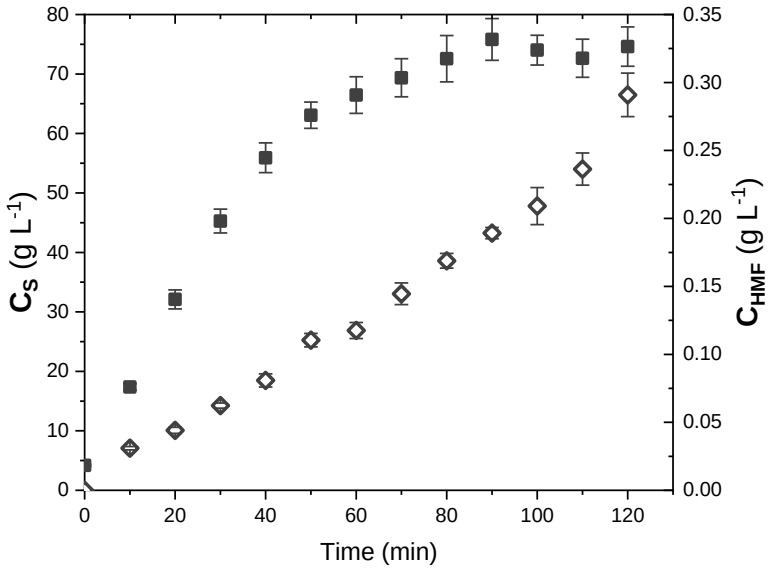

Figure 1. Evolution over time of the concentration of substrate (S), glucose in this case, and of HMF during the acid hydrolysis of potato residues. Data points: ■ substrate; ◇ HMF.

3.2. Production of succinic acid in bottles from glucose and potato hydrolysate

As a first test, succinic acid was produced both from pure glucose (40 g L^{-1}) and from potato acid hydrolysate with a similar concentration of glucose using anaerobic conditions and closed bottles as indicated in section 2.2. Results are collected in Table 1, where it is observed that the outcome regarding acid final titers and yields are really similar, while productivity decreases by 30% and selectivity to SA is also reduced by 16% when replacing a pure glucose medium by potato hydrolysate.

Table 1. Succinic acid concentration, yield, productivity and selectivity in its production process from pure glucose and potato hydrolysate in bottles.

Substrate	Pure glucose	Potato hydrolysate
$C_{P.max}$ (g L^{-1})	11.3	10.6
$Y_{S,0}$ (g g^{-1})	0.28	0.27
$Y_{S,cons}$ (g g^{-1})	0.53	0.50
Prod. (g g^{-1} L^{-1})	0.46	0.32
Sel. (g g^{-1})	0.55	0.46

3.3. Production of succinic acid in a bioreactor from different initial concentrations of glucose and development of a simple kinetic model

In Figure 2, it can be observed that there are similar biomass temporal curves for all glucose concentrations, with identical maximal biomass concentration in the stationary phase of 5-5.5 g L^{-1} and equivalent slope values at the inflection points in the exponential phase of the growth curves (maximal biomass production rate). Likewise, substrate consumption curves are almost lines, except for the lag phases, that have practically the

same slopes except for the 50 g L^{-1} glucose experiment, where the consumption rate is slightly slower. Very similar trends in the production rate of succinic acid are appreciated, again except for the 50 g L^{-1} experiment, where SA production starts 1-2 h later at a slower pace, suggesting an incipient substrate inhibition. Finally, the lower the glucose concentration the higher the rate of by-product generation is.

Similar final by-product values are achieved regardless of the initial biomass concentration, with an increase in selectivity with the initial glucose concentration, from 0.5 g g^{-1} at 20 g L^{-1} to 0.7 g g^{-1} at 50 g L^{-1} of substrate.

Low concentrations of the carbon source were favorable both for the yield and for the productivity of succinic acid, with values of 0.72 g g^{-1} and 0.98 g L^{-1} h^{-1} for the runs at 20 g L^{-1} and 0.68 g g^{-1} and 0.71 g L^{-1} h^{-1} when working with 50 g L^{-1} of glucose.

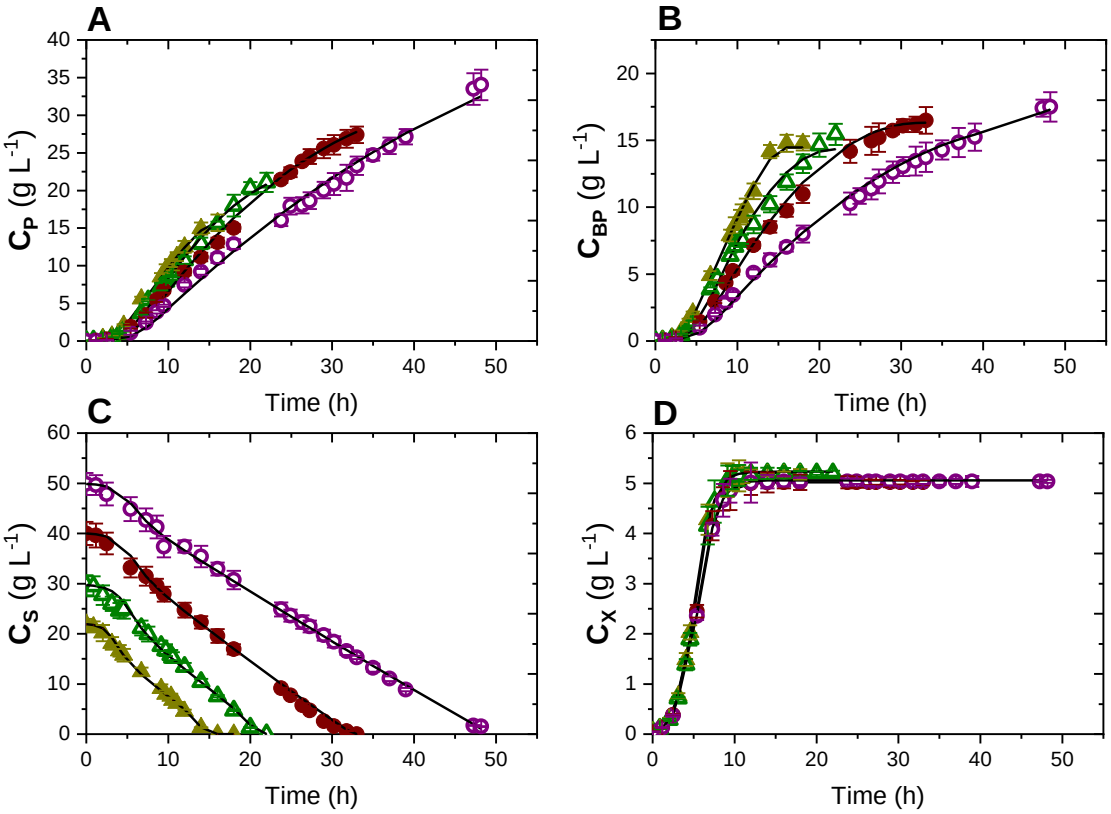

Figure 2. Kinetic modeling of succinic acid production in runs at different initial glucose concentrations. Data points: ▲ run 1 – 20 g· L^{-1} glucose; △ run 2 – 30 g· L^{-1} glucose; ● run 3 – 40 g· L^{-1} glucose; ○ run 4 – 50 g· L^{-1} glucose. Model predictions shown as lines.

Comparing the results of this study with those obtained by other authors, it is evident that, at the moment, there is no consensus regarding the production trends of succinic acid from glucose in batch-type operations with *A. succinogenes*. (Luthfi et al., 2018) observed an inhibition of cell growth as the initial glucose concentration increased from 20.3 g L^{-1} to 123.1 g L^{-1}. They achieved optimum yield and productivity (0.62 g g^{-1}, 2 g L^{-1} h^{-1}) when fermenting from 60.3 g L^{-1} of substrate. Above that concentration runs seemed to suffer from some kind of inhibition by substrate. In addition, they observed a decrease in

selectivity as the initial amount of glucose in the culture medium increased. On the other hand, Ferone et al. studied the influence of the initial concentration of this sugar in a range between 5.4 and 80.7 g L^{-1} (Ferone et al., 2017). Although the yield results showed oscillations, a downward trend could be deduced as the initial amount of the carbon source increased, coinciding with this study. Despite this, they established a concentration of 42.7 g L^{-1} of glucose as the optimum value, a value at which they observed the optimal values of productivity and succinic acid/acetic acid ratio (0.36 g L^{-1} h^{-1}, 0.74 g g^{-1}). It is also worth noting the work of (Salvachúa et al., 2016) who studied the effect of the initial glucose concentration between 40 g L^{-1} and 100 g L^{-1} . Although the maximum concentration of glucose was reached at 100 g L^{-1}, productivity was clearly impaired from 80 g L^{-1} of substrate, a concentration at which there was also no complete consumption of this sugar.

Considering data collected in Figure 2, we have applied a modified version of a kinetic model published elsewhere (Escanciano et al., 2023). This model is based in a simple reaction scheme based on the logistic equation –equation 11- for the biomass growth and potential kinetic equations to explain substrate consumption and product and byproducts onsets. This kinetic model is of the unstructured non-segregated type, considering the bacteria as a single component (no differences between diverse bacterial cells) and only major substrates and products outside the cells. The reaction network is presented through a scheme depicting a first reaction considering glucose (S) consumption (r1, equation (8)) to obtain biomass X, another reaction r2 –equation (9)- to explain SA generation (P) and a final reaction r3 –equation (10)- that describes byproduct (BP) creation from glucose. From this scheme, the kinetic equation for the corresponding reaction rates is displayed in equations (11) to (13). The logistic equation describes the microorganism growth based on two relevant kinetic parameters: the maximum value of the biomass concentration – C_{Xm}- and the specific growth rate -μ-. The potential equations describing the evolution of substrate, SA and by-products are independent of the logistic equation, and are characterized by the kinetic constants k_{P1} for SA and k_{P2} for the sum of the by-products. Finally, several macroscopic yields link biomass, substrate, SA and by-products: $YS_{/X}$, $Y_{S/P1}$, $Y_{S/BP}$ and $Y_{S/P2}$ in equations (14) to (17).

Reaction network

$$Y_{S/X} S \xrightarrow{r_1} X \tag{8}$$

$$Y_{S/P1} S \xrightarrow{r_2} P + Y_{S/BP} \cdot BP \tag{9}$$

$$Y_{S/P2} S \xrightarrow{r_3} BP \tag{10}$$

Reaction rates

$$r_1 = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_{Xm}}\right) \tag{11}$$

$$r_2 = k_{P1} \cdot C_S \cdot C_X \tag{12}$$

$$r_3 = k_{P2} \cdot C_S \cdot C_X \tag{13}$$

Production and consumption rates

$$R_S = \frac{dC_S}{dt} = -Y_{S/X} \cdot r_1 - Y_{S/P1} \cdot r_2 - Y_{S/P2} \cdot r_3 \tag{14}$$

$$R_P = \frac{dC_P}{dt} = r_2 \quad (15)$$

$$R_X = \frac{dC_X}{dt} = r_1 \quad (16)$$

$$R_{BP} = \frac{dC_{BP}}{dt} = Y_{S/BP} \cdot r_2 + r_3 \quad (17)$$

Figure 2 shows how the model fit very accurately to all relevant data, with very low RMSE values (0.58 – 1.28) and very high values for the estimated Fisher's F (20,191 – 41,242). Likewise, VE values are high, near 100% (97.8 % - 99.5 %). Table 2 compiles the values retrieved for the kinetic constants, the maximum biomass concentrations and the macroscopic yields.

Until now, most of the kinetic models developed are of the unstructured-non-segregated type, being limited, moreover, to the prediction of the evolution of biomass or succinic acid. However, some authors, such as (Li et al., 2022; Lin et al., 2008; Pateraki et al., 2016; Song et al., 2008; Vlysidis et al., 2011) have proposed models that allow studying the kinetics of biomass, succinic acid and by-products simultaneously in the fermentation carried out by different microorganisms (*A. succinogenes*, *Basfia succiniproducens*, *Mannheimia succiniproducens* and *Yarrowia lipolytica*). The biomass formation rate in these studies was based on a Monod type model, considering, in addition, that it could be susceptible to substrate inhibitions of the Haldane-Andrews type and/or Luong type and product inhibitions of the Luong type. In addition, they agreed on the decision to include the Pirt's maintenance coefficient in the rate of the carbon source consumption equations and on the use of the Luedeking-Piret expression to estimate the evolution of succinic acid and by-products concentrations (Escanciano et al., 2022b).

These models are highly accurate and take into account a large number of inhibitions due to substrate or product, however, they imply the need to incorporate a large number of parameters, needing to estimate up to 19 kinetic parameters. This is quite inconvenient from the point of view of chemical engineering, due to its possible application in carrying out process scaling or techno-economic analysis. Therefore, it is necessary to limit the use of these parameters as much as possible (Escanciano et al., 2022b; Li et al., 2022; Lin et al., 2008; Pateraki et al., 2016; Song et al., 2008; Vlysidis et al., 2011).

In all cases, the estimation of the Pirt's coefficient obtained extremely low values (Li et al., 2022; Song et al., 2008; Vlysidis et al., 2011) and, since, as previously discussed, there is a wide range of initial substrate concentration values that do not generates inhibition (Ferone et al., 2017; Luthfi et al., 2018; Salvachúa et al., 2016), on many occasions sugar consumption can be estimated without the need for this type of parameter. Regarding the inhibition by products and by-products, (Lin et al., 2008), authors who worked with *A. succinogenes* and glucose as a carbon source, as in the present study, observed that formic acid was the compound that generated the greatest inhibition in production of succinic acid, determining that the critical concentrations for formic acid, ethanol, acetic acid, pyruvic acid and succinic acid would be, respectively: 16 g L^{-1}, 42 g L^{-1}, 46 g L^{-1}, 74 g L^{-1} and 104 g L^{-1}.

In this work, no statistically significant variations have been observed depending on glucose initial concentrations for most of the estimated kinetic parameters, except for k_{P1}. This kinetic constant, as shown in Figure 3, clearly decreases as the substrate concentration increases, confirming the substrate inhibition first appreciated in Figures 2A and 2C. Therefore, for the approach of a model that would take this parameter into account, which affects both the rate of formation of products and by-products, and which does not drastically increase the number of kinetic parameters, equation (18) was proposed. Thus this parameter is calculated as a function of an exponential equation that incorporates the constants α and β, allowing the simultaneous adjustment of all the experiments to different initial glucose concentrations (Table 2).

$$k_{P1} = \alpha \cdot e^{-\beta \cdot C_{S0}} \tag{18}$$

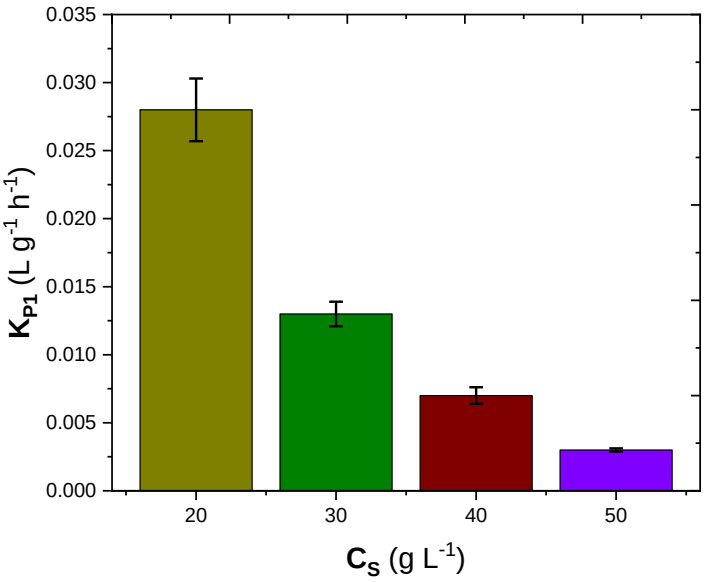

Figure 3. K_{P1} constant as a function of substrate concentration

Table 2. Kinetic parameter values calculated by fitting the kinetic model to experimental data of succinic acid production from different initial glucose concentrations individually and together

C_S (g L^{-1})	20			30			40			50			All runs		
C_{Xm} (g L^{-1})	5.209	±	0.019	5.232	±	0.032	5.019	±	0.021	5.060	±	0.045	5.095	±	0.027
kp_1 (L· g^{-1}· h^{-1})	0.028	±	0.002	0.013	±	0.001	0.007	±	0.001	0.003	±	0.0001			
kp_2 (L· g^{-1}· h^{-1})	0.017	±	0.002	0.017	±	0.004	0.018	±	0.002	0.018	±	0.001	0.017	±	0.001
μ (h^{-1})	0.859	±	0.015	0.854	±	0.023	0.845	±	0.043	0.839	±	0.025	0.923	±	0.016
$Y_{S/P1}$ (g g^{-1})	0.818	±	0.023	0.891	±	0.022	0.823	±	0.062	0.888	±	0.020	0.830	±	0.066
$Y_{S/P2}$ (g g^{-1})	1.696	±	0.310	1.625	±	0.109	1.773	±	0.132	1.646	±	0.157	1.667	±	0.089
$Y_{S/BP}$ (g g^{-1})	1.138	±	0.149	1.112	±	0.111	1.099	±	0.091	1.194	±	0.091	1.102	±	0.043
$Y_{S/X}$ (g g^{-1})	1.174	±	0.129	1.632	±	0.141	1.115	±	0.082	1.532	±	0.136	1.120	±	0.087
α (L· g^{-1}· h^{-1})													0.152	±	0.008
β (L·g^{-1})													0.079	±	0.001

Table 3. Statistical parameter values calculated by fitting the kinetic model to experimental data of succinic acid production from different initial glucose concentrations individually and together

C_S (g L^{-1})	F_{95}	RMSE	SSR	VE %
20	30616	0.84	7.06	99.3
30	29587	0.87	8.76	98.6
40	41242	0.67	6.47	99.5
50	33646	0.58	7.70	99.1
All runs	20191	1.28	9.63	97.8

3.4. Production of succinic acid in a bioreactor from potato hydrolysate

Regarding the production of succinic acid, Table 4 shows a notably higher final product concentration when potato hydrolysate is used as a carbon source instead of commercial glucose. The experiment based on the hydrolysate increases the yield to SA by 37% compared to the experiment with pure glucose, also the selectivity by 22.5% (reaching a value of 0.80 g g^{-1}), which is even more relevant. However, productivity decreases by 24%, probably due to the presence of important amounts of HMF. Table 4 summarizes the production results obtained in this study together with those obtained by other authors from biomass in recent years.

Among the experiments for the production of succinic acid from biomass, it is worth highlighting the work of (Marinho et al., 2016), who achieved a productivity of 3.90 g L^{-1} h^{-1} with a batch operation from macroalgae *Saccharina latissima*, one of the highest productivities achieved from a non-commercial carbon source. (Thuy et al., 2017) carried out the fermentation process of fresh cassava root with a very high production speed (3.22 g L^{-1} h^{-1}) through a fed-batch type operation. In addition, these same authors were the only ones that managed to exceed the yield of this study (0.92 g g^{-1}), reaching a reaction yield of 1.51 g g^{-1}.

In recent years, the number of studies focused on the production of succinic acid from food residues has been increasing (Morone et al., 2019; Sayury Nishida et al., 2021). (Filippi et al., 2021) used grape pomace and stalks in fed-batch operation, achieving a yield of 67 % and a productivity of 0.79 g L^{-1} h^{-1}. This same type of operation was used by (Oreoluwa Jokodola et al., 2022) from olive stone, but limited to a yield of 27% and a productivity of 0.5 g L^{-1} h^{-1}. (Lo et al., 2020)carried out a batch type fermentation with the same microorganism to produce succinic acid from sweet sorghum bagasse, achieving yields of 61 % and productivities of 0.89 g L^{-1} h^{-1}. The use of citrus residues has also been studied, as in the case of (Patsalou et al., 2020), who operating in fed-batch with this carbon source obtained relatively low productivity values (0.45 g L^{-1} h^{-1}), but they did obtain competitive yield values (0.73 g g^{-1}). Although one of the highest yields of succinic acid from food residues (0.92 g g^{-1}) has been achieved in the present study, other authors have managed to achieve considerably higher productivities, such as (Corona-González et al., 2016) (1.32 g L^{-1} h^{-1}), thanks to the fermentation of Tequilana agave bagasse by discontinuous operation, though obtaining considerably lower yield values (0.39 g g^{-1}).

Table 4. Comparison of the bibliographic results of succinic acid production from biomass through the action of *A. succinogenes* with those corresponding to this study in a reactor using glucose and potato residues as substrate.

Type of operation	Substrate	C_P (g· L^{-1})	$Y_{S.0}$ (g· g^{-1})	Prod. (g· L^{-1}· h^{-1})	Reference
Fed-batch	Grape pomace and stalks	40.2	0.67	0.79	(Filippi et al., 2021)
Repeated batch	Tequilana agave bagasse	33.6	0.39	1.32	(Corona-González et al., 2016)
Fed-batch	Olive pits	33.7	0.27	0.50	(Oreoluwa Jokodola et al., 2022)
Batch	Napier grass	17.5	0.58	0.79	(Lee et al., 2022)
Fed-batch	Citrus peel waste	22.4	0.73	0.45	(Patsalou et al., 2020)
Batch	Palm oil hydrolysate	36.5	0.57	1.95	(Luthfi et al., 2018)
Batch	Grape must	88.9	0.66	0.93	(Hijosa-Valsero et al., 2022)
Batch	Sweet sorgum bagasse	17.8	0.61	0.89	(Lo et al., 2020)
Batch	Sugarcane juice	57.9	0.89	1.21	(Shen et al., 2016)
Batch	Macroalgae *Saccharina latissima*	36.8	0.92	3.90	(Marinho et al., 2016)
Batch	Macroalgae *Laminaria digitata*	24.4	0.86	0.50	(Alvarado-Morales et al., 2015)
Fed-batch	Fresh cassava root	151	1.51	3.22	(Thuy et al., 2017)
Batch	Glucose – 20 g L^{-1}	15.7	0.72	0.98	This study
Batch	Glucose – 30 g L^{-1}	21.1	0.71	0.96	This study
Batch	Glucose – 40 g L^{-1}	27.4	0.68	0.83	This study
Batch	Glucose – 50 g L^{-1}	34.0	0.67	0.71	This study
Batch	Potato wastes	32.2	0.92	0.64	This study

3.5. Estimation of kinetic parameters in fermentation with potato residues

Figure 4 shows the evolution of the substrate, succinic acid, by-products (acetic and formic acids) and biomass in the experiment carried out in a reactor using a hydrolysate of potato waste as a carbon source. This figure also represents the fit to the experimental data of the kinetic model developed for pure glucose in section 3.3.

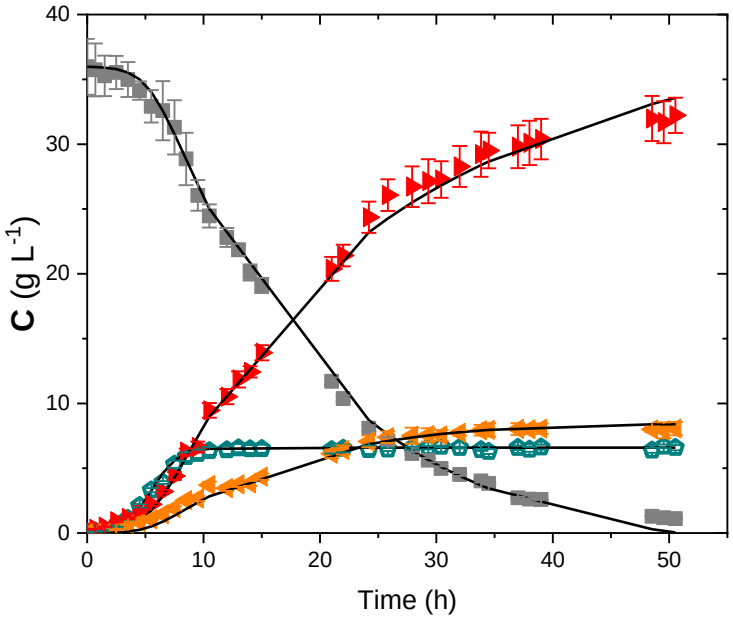

Figure 4. Kinetic modeling of succinic acid production employing potato wastes as carbon source. Data points: ■ observed substrate; ▶ observed product; ◀ observed by-products; ⬠ observed biomass. Model predictions shown as lines

Likewise, we can observe in Figure 4 how the fitting of the model (in lines) is very accurate. Again, RMSE values are low, while F-values are much higher than the values needed to overcome the null hypothesis at 95% confidence (threshold F-value interval tabulated at 95% confidence for the relevant numerator and denominator degrees of freedom: 20-30). Moreover, VE values are near 100%, so the kinetic model easily explains the evolution of all relevant dependent variables with process time. As an example, in Table 5, the overall values for F, VE and RMSE, as well as for the Sum of Squared Residuals (SSR) are collected. In this Table, the values of the kinetic parameters are also compiled together with their intervals of error at 95 % confidence according to the Student test, showing that the values, as happened in the case of using pure glucose as carbon source or substrate, are statistically significant.

The comparison of the results, obtained with potato hydrolysate and with pure glucose in terms of the values of the corresponding kinetic parameters, shows that there is a higher final biomass concentration (6.587 g L^{-1} vs. 5.095 g L^{-1}) when using the potato hydrolysate, but, at the same time, we appreciate a lower growth rate (μ_m is 0.878 h^{-1} versus 0.923 h^{-1}), showing a higher need, and ability, for the microorganism to adapt to the hydrolysate, probably due to the presence of toxic substances like HMF or the phenolic compound present in potato and potato processing-derived waters and solutions (Akyol et al., 2016). The increase in succinic acid yield is justified due to the increase in Y_{SP1} (1.337 g g^{-1} vs. 0.830 g g^{-1} with pure glucose). Very interestingly, less by-product generation is reflected in $Y_{S/BP}$ and Y_{SP2}, which dramatically decrease from 1.102 g g^{-1} and 1.667 g g^{-1} with pure glucose to 0.363 g g^{-1} and 0.585 g g^{-1} with the hydrolysate. In

addition, k_{p2} also decreases from 0.017 $L \cdot g^{-1} \cdot h^{-1}$ with commercial glucose to 0.007 $L \cdot g^{-1} \cdot h^{-1}$with the hydrolysate. The parameters of k_{p1} only undergo variations within the confidence interval.

Table 5. Kinetic and statistical parameter values calculated by fitting the kinetic model to experimental data of succinic acid production employing potato wastes as carbon source.

C_{Xm} (g L^{-1})	6.587	±	0.043	**F_{95}**	30473
kp_2 ($L \cdot g^{-1} \cdot h^{-1}$)	0.007	±	0.0003	**RMSE**	0.88
μ (h^{-1})	0.878	±	0.042	**SSR**	6.18
$Y_{S/P1}$ (g g^{-1})	1.337	±	0.066	**VE (%)**	98.4
$Y_{S/P2}$ (g g^{-1})	0.585	±	0.045		
$Y_{S/BP}$ (g g^{-1})	0.363	±	0.018		
$Y_{S/X}$ (g g^{-1})	0.415	±	0.015		
α ($L \cdot g^{-1} \cdot h^{-1}$)	0.157	±	0.001		
β ($g \cdot L^{-1}$)	0.073	±	0.001		

4. Conclusions

From fermentation experiments with *A. succinogenes* at different initial glucose concentrations, it has been possible to apply a precise kinetic model of succinic acid production, by-products, biomass and substrate consumption in an easy way. Subsequently, after verifying the possibility of producing succinic acid from potato wastes in bottles, this kinetic model was applied to the production from these residues in a reactor, through a batch operation. In this last production it was possible to obtain one of the highest succinic acid bioproduction yields from food residues that can be found in the bibliography (0.92 g g^{-1}), even surpassing its equivalent operation with pure glucose (0.68 g g^{-1}). However, when commercial sugar was used, the rate of succinic acid production was higher (0.83 g L^{-1} h^{-1}) than when the hydrolysate was used as a carbon source (0.64 g L^{-1} h^{-1}). This study shows the potential of potato waste reuse as part of the circular economy concept to develop solutions based on the biorefinery approach.

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Declaration of Competing Interest

The authors declare that they have no knowledge of competing financial interests or personal relationships that could have influenced or appeared to influence the work here reported.

Prof. Dr. Run-Cang Sun

Editor-in-Chief Industrial Crops and Products

Madrid, 19th March 2023

Dear Prof. Dr. Run-Cang Sun

Attached to this letter you may find files containing the manuscript, figures and tables, and highlights of a research paper entitled "Bioproduction of succinic acid from potato waste: process development and kinetic modeling" that we wish to publish in Industrial Crops and Products. The authors are Itziar A. Escanciano, Victoria E. Santos, Angeles Blanco and Miguel Ladero. All authors have agreed to publish this review in Industrial Crops and Products, being this the first time this manuscript is submitted for publication.

The paper focuses on the production of succinic acid from pure glucose solutions as model growth/production media and the same production in glucose-rich acid hydrolysates from potato waste treatment. First, a set of runs for succinic acid production was performed in bottles ensuring anaerobic conditions, following the temporal evolution of the substrate, the targeted acid and the by-products, together with biomass. Secondly, the acid hydrolysis of a potato puree coming from industrial wastes was performed at 100 °C using a 1 M HCl media; we choose a 60 min treatment to maximize the ratio glucose/5-HMF. Afterwards, several runs in closed stirred bottles and in a 2.5 L biorreactor were performed with this hydrolysate. Finally, a proposed kinetic model was successfully applied to the fermentation data obtained from potato waste fermentation and from model glucose solutions, observing a notable substrate inhibition as indicated by the K_{p1} decreasing value with increasing initial glucose concentration. At 30-40 g L^{-1}, it can be observed that the use of potato waste hydrolysate is notably more effective than similar pure glucose concentration media, in terms of succinic acid final concentrations, with notable enhancements of productivity and yield to the acid. In fact, with the hydrolysate we could produce 32.2 g L^{-1} of succinic acid with a productivity of 0.64 g L^{-1} h^{-1}, improving the yield by 35% compared to fermentation with pure glucose, while we obtained one of the highest bibliographic values for succinic acid bioproduction from food residues (0.92 g g^{-1}).

Hoping the contents of this manuscript fulfils the standards and expectations of Industrial Crops and Products, I remain sincerely yours.

Miguel Ladero

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Highlights

- High yield production of bio-succinic acid is achieved by *Actinobacillus succinogenes*
- Succinic acid production is viable using potato residues
- Higher yields have been achieved with potato residues than with pure glucose
- An unstructured non-segregated kinetic model is valid at several glucose concentrations
- This kinetic model has been successfully applied to bioprocesses with potato residues

CRediT author statement

Itziar A. Escanciano: Methodology, Software, Investigation, Data curation, Writing-original draft preparation, Visualization.

Victoria E. Santos: Conceptualization, Software, Validation, Formal analysis, Resources, Data curation, Writing—review and editing, Visualization, Supervision, Funding adquisition.

Ángeles Blanco: Conceptualization, Validation, Resources, Writing—review and editing, Visualization, Supervision, Project administration, Funding adquisition.

Miguel Ladero: Conceptualization, Validation, Formal analysis, Resources, Data curation, Writing—review and editing, Visualization, Supervision, Project administration, Funding adquisition.

8.6 PUBLICACIÓN 6

Autores: Itziar A. Escanciano, Ángeles Blanco, Victoria E. Santos y Miguel Ladero

Título: Integral use of brewery wastes as carbon and nitrogen sources for the bioproduction of succinic acid

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Categoría: Ingeniería Agrícola, Biotecnología y Microbiología Aplicada, Energía y Combustibles

Resumen:

La bioeconomía circular es uno de los principales objetivos socioeconómicos del siglo XXI, que incluye el uso de residuos de biomasa y su transformación mediante procesos respetuosos con el medio ambiente en bloques de construcción de biorrefinería. Entre estos compuestos destaca el ácido succínico (AS) obtenido por fermentación. Este trabajo demuestra la viabilidad de utilizar bagazo de cerveza y levadura de cerveza gastada como fuentes de carbono y nitrógeno para la bioproducción de AS con *Actinobacillus succinogenes*. Un tratamiento enzimático progresivo liberó monosacáridos y péptidos simples que fueron utilizados por el microorganismo, en una fermentación posterior. En comparación con el uso de xilosa comercial y extracto de levadura, el uso de residuos de cerveza obtuvo mejores rendimientos y selectividad, aunque con una productividad ligeramente menor. Finalmente, se ajustó con éxito un modelo cinético no estructurado y no segregado, facilitando la futura realización de análisis tecnoeconómicos, escalado del proceso o diseño de un sistema de control.

Bioresource Technology

Integral use of brewery wastes as carbon and nitrogen sources for the bioproduction of succinic acid

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Abstract:	Circular bioeconomy is one of the major socio-economic objectives for the 21st century, which includes the use of biomass waste and its transformation through environmentally friendly processes into biorefinery building blocks. Among these compounds, succinic acid (SA) obtained by fermentation stands out. This work demonstrates the feasibility of using beer bagasse and spent brewer's yeast as carbon and nitrogen sources for the bioproduction of SA with Actinobacillus succinogenes. The use of a progressive enzymatic treatment liberated simple monosaccharides and peptides that were used by the microorganism, in a subsequent fermentation, to produce up to 14 g L-1 SA. Compared to the use of commercial xylose and yeast extract, the used of beer wastes obtained better yields and selectivity, though with a slightly lower productivity. Finally, an unstructured non-segregated kinetic model was successfully fitted, facilitating the future performance of techno-economic analyses, scaling of the process or design of a control system.
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Prof. **Ashok Pandey**

Editor-in-chief of Bioresource Technology

Dear Professor Pandey,

Allow me to introduce myself, my name is Victoria E. Santos, I am a professor in the Department of Chemical Engineering and Materials of the Faculty of Chemical Sciences of the Complutense University of Madrid. I am sending you an original article entitled '***Integral use of brewery wastes as carbon and nitrogen sources for the bioproduction of succinic acid'*** which has been carried out by Itziar A. Escanciano (Bachelor and Master in Chemical Engineering at UCM), Prof. Dr. María Ángeles Blanco, professor Miguel Ladero (both same department and university as me) and I.

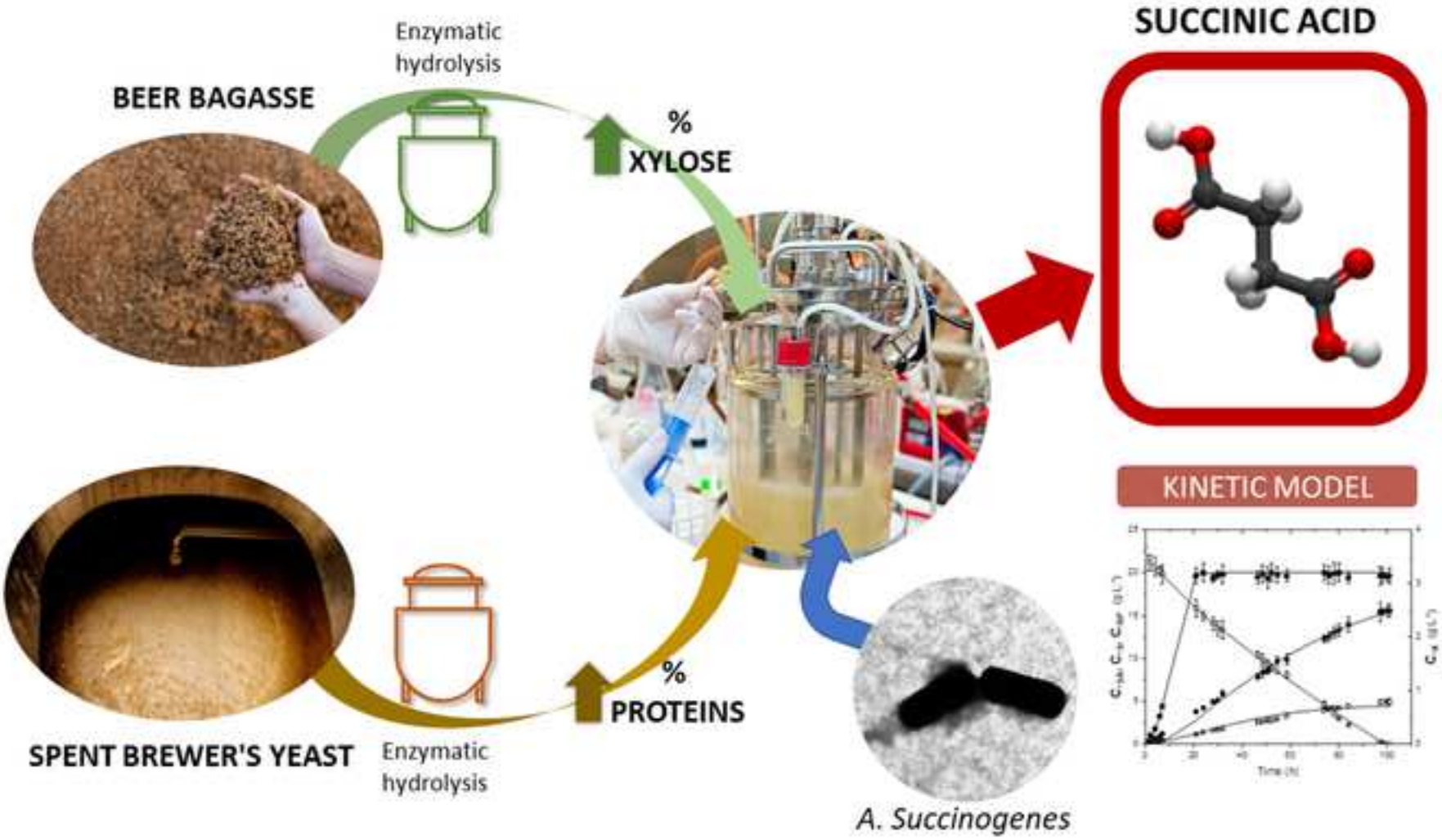
As you know, in 2004, the U.S. Department of Energy began to identify molecules of industrial interest that could be synthesized from sugars (building blocks). The raw material that is presented as the best candidate is biomass, being especially interesting that which is considered as waste (pruning residues, cereal straw, agri-food waste, ...). Generally, the fraction used is the cellulose fraction because from its hydrolysis glucose is obtained, a sugar perfectly suited to microbial metabolisms. However, the hemicellulose fraction presents problems because the predominant sugar is xylose, generally not assimilable by the metabolism of microorganisms. Most of the studies carried out on the production of building blocks focus on glucose as a substrate. However, in order to achieve the integral utilization of the type of "waste" mentioned above, it is of vital importance to develop technologies capable of using sugars such as xylose, so it is necessary to identify, develop and fine-tune production processes based on xylose.

The manuscript I am sending you for possible publication in Bioresource Technology is an original work focused on the microbial production of one of the main building blocks: succinic acid. Most of the published works on the fermentative production of this molecule have focused on the use of glucose as substrate. However, very few studies have focused on the valorization of the hemicellulose fraction. This work is focused on the use of xylose, that is the main sugar in beer bagasse. Nevertheless, the aim of this work is to demonstrate the feasibility of using not only beer bagasse but also spent brewer's yeast as carbon and nitrogen sources for the bioproduction of succinic acid with Actinobacillus succinogenes. I would also like to highlight the kinetic modeling carried out, using a simple model that will be very useful both for techno-economic studies and for the design of bioreactors. I have chosen the classification BIOPROCESSES, which includes submerged fermentation, kinetics, modeling and optimization of bioprocesses.

This manuscript aims with the SDGs: 9, 11, 12, 13 and 15. I hope it will be of interest to you and *Bioresource Technology.*

Sincerely

Victoria E. Santos



Highlights

- Bioproduction of succinic acid from brewery residues
- Beer bagasse was the carbon source and spent brewer's yeast the nitrogen source
- Production with waste and commercial xylose and yeast extract were compared
- Production from beer bagasse and spent brewer’s yeast obtained the highest yield
- A simple and accurate kinetic model could be applied in the fermentation from waste

Integral use of brewery wastes as carbon and nitrogen sources for the bioproduction of succinic acid

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Abstract

Circular bioeconomy is one of the major socio-economic objectives for the 21st century, which includes the use of biomass waste and its transformation through environmentally friendly processes into biorefinery building blocks. Among these compounds, succinic acid (SA) obtained by fermentation stands out. This work demonstrates the feasibility of using beer bagasse and spent brewer's yeast as carbon and nitrogen sources for the bioproduction of SA with *Actinobacillus succinogenes*. The use of a progressive enzymatic treatment liberated simple monosaccharides and peptides that were used by the microorganism, in a subsequent fermentation, to produce up to 14 g L^{-1} SA. Compared to the use of commercial xylose and yeast extract, the used of beer wastes obtained better yields and selectivity, though with a slightly lower productivity. Finally, an unstructured non-segregated kinetic model was successfully fitted, facilitating the future performance of techno-economic analyses, scaling of the process or design of a control system.

Keywords: succinic acid; brewery wastes; *Actinobacillus succinogenes*; kinetic modeling, circular economy

1. Introduction

Currently, the largest beer consumers are the US, China, Brazil, Russia and Germany. Although the largest percentage of beer produced still comes from large companies, numerous craft beer businesses have been promoted since 1970, becoming a sector that has gaining an important market power, especially since the COVID-19 pandemic began, at which time drinking habits underwent severe changes. Despite the fact that in the developed regions the consumption of beer remained constant or even in decline, in the 21st century there has been a large increase in beer consumption globally due to the impact on its demand from developing regions and, therefore, intensifying the amount of waste generated by this growing industry (Pokrivčák et al., 2019; Size, Dental Implants Market, 2018; TBOE, 2021). Beer bagasse, also known as brewer's spent grain, amounts to 85% of the total solid waste in brewery, with a total yearly production of 34-35 million tons in Europe, while brewer's spent yeast is obtained at a rate of 0.125 million tons/year (Karlović et al., 2020).

The generation of food waste is a problem that is gaining more and more prominence. According to the Food and Agriculture Organization (FAO), each year 1,300 million tons of food are wasted, including in these figures both the production chain and its distribution and consumption ((FAO), n.d.; Esteban and Ladero, 2018). The lack of efficiency in the use of food resources jeopardizes the fulfillment of several of the Sustainable Development Goals (SDGs), among which it is worth highlighting: end hunger, achieve food security and improved nutrition and promote sustainable agriculture (SDG 2); build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation (SDG 9); ensure sustainable consumption and

production patterns (SDG 12); and take urgent action to combat climate change and its impacts (SDG 13) (Baeyens and Goffin, 2015).

Biorefineries represent a great solution to part of this problem and, therefore, meet several of these objectives. In this case, the circular economy process is based on the use of sugars, oils and proteins from food wastes to obtain biofuels, chemicals and materials such as plastics and polymers, through biotechnological processes (Teigiserova et al., 2019). According to the United States Department of Energy (US DOE), succinic acid is one of the 12 main platform chemicals (Werpy and Petersen, 2004) (Dienst and Onderzoek, 2015). Traditionally, succinic acid has been used for the production of resins, coatings, and pigments. It also has many applications in the food industry as an acidulant, flavoring and sweetener, as well as in the pharmaceutical industry. It is worth noting its great potential as a replacement for maleic anhydride, acting as a chemical platform for the generation of a multitude of compounds. Furthermore, one of its most promising applications in the bioeconomy era is the production of biodegradable polymers, such as polyamides and polyesters (Escanciano et al., 2022b; Mancini et al., 2022; Oreoluwa Jokodola et al., 2022a).

Since succinic acid is an intermediate compound of the Tricarboxylic Acid Cycle (TCA), it can be synthesized by almost all cells, both plant and animal. The use of fungi for the production of this compound has been extensively studied, however, the use of these microorganisms presents numerous difficulties both during fermentation and in the separation and purification processes. To date, bacteria isolated from the rumen of cattle are considered the best candidates to produce this acid, being its final product during anaerobic fermentation. The most promising strain is *Actinobacillus succinogenes*, a bacterium with the distinctive ability to produce a relatively large

amount of succinic acid under anaerobic conditions from a wide variety of carbon sources such as arabinose, cellobiose, fructose, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose or xylose (Ferone et al., 2019; Liu et al., 2022; Pateraki et al., 2016).

In recent years, great efforts have been devoted to the investigation of succinic acid production from residues through fermentation processes (Table 1) (Chiang et al., 2021; Jiang et al., 2017; Narisetty et al., 2022; Wang et al., 2022; Xu et al., 2021). It is worth noting the work of (Filippi et al., 2022), in which they used winery wastes (grape pomace, stalks and wine lees) to produce bacterial cellulose, value-added fractions and succinic acid; they worked with *A. succinogenes* as a biocatalyst for the production of this last compound. (Oreoluwa Jokodola et al., 2022a) also used this microorganism and olive pits and sugarcane bagasse as carbon sources, two residues rich in xylose. (Hijosa-Valsero et al., 2022) compared the performance of *A. succinogenes* and *Basfia succiniproducens* in the production of succinic acid from vine shoots and surplus grape and, although both species had similar yields from vine shoots, fermentation from grape with *A. succinogenes* obtained much better results than with *B. succinoproducens*. Despite its high costs, as a general rule, in the production of succinic acid, yeast extract (YE) (Escanciano et al., 2022b; Ferone et al., 2019; Hijosa-Valsero et al., 2022; Lee et al., 2022) is the usual source of nitrogen (Alvarado-Morales et al., 2015), although its substitution by corn steep liquor (CSL) has also been studied. (Tan et al., 2016; Xi et al., 2013) as well as the mixture of both nutrient sources (Cao et al., 2018). (Jiang et al., 2010) used brewer's spent yeast for the production of succinic acid from glucose. They compared the effect of brewer's yeast pretreatment by autolysis or by enzymatic hydrolysis, observing better fermentation performance from the second hydrolysate.

However, to achieve complete glucose consumption, they needed to supplement the medium with vitamins. In summary, although the state of the art shows the feasibility of producing succinic acid by biotechnological processes, it has a high cost mainly associated to the cost of the carbon source and, specially, of the nitrogen source.

Therefore, this paper is focused on producing succinic acid by biotechnological means at a lower cost by promoting circular economy concepts. The novelty is based on using brewery wastes as source of secondary raw materials to replace both carbon and nitrogen sources in the fermentation process. Beer bagasse, rich in xylose, is used as carbon source and brewer's spent yeast as nitrogen source, without vitamin supplementation. The results are compared with fermentations carried out using commercial xylose and yeast extract. Finally, a kinetic model will be fitted to experimental data on the evolution of the species involved in the fermentation carried out from hydrolysates. facilitating the future performance of techno-economic analyses, scaling of the process or design of a control system.

2. Materials and methods

2.1. Beer bagasse hydrolysis

Beer bagasse, obtained from a local brewery (La Cibeles S.L - Madrid, Spain) was dried at 40 ºC for 48 h, ground and sieved until reaching a size of 0.75 mm. Afterwards, it was mixed with water in a 1:6 w:v ratio and adjusted to pH 5.5 with H_3PO_4. Subsequently, enzymatic hydrolysis was carried out in 3 steps. The first was carried out at 90 ºC and 180 rpm for 1 h, adding 1 mL of Termamyl® SC DS for each liter of the mixture of water and bagasse. In the next stage, the temperature was lowered to 55 ºC and hydrolysis was carried out for 1 h at 180 rpm with the addition of 0.3 mL L^{-1}

Saczyme® Yield, 0.3 mL L^{-1} FAN Boost™, and 0.3 mL L^{-1} Ultraflo® XL. In the last stage, the pH was adjusted to 5 (H_3PO_4) and the sample was hydrolyzed for 10 h at 180 rpm and 45 ºC with 15 mL L^{-1} of Celluclast® 1.5L (Djukić-Vuković et al., 2016; Rojas-Pérez et al., 2022). The obtained hydrolysate was centrifuged for 20 min at 4 ºC and 8,000 rpm. Due to the high amount of volatiles in the hydrolysate (Castilla-Archilla et al., 2021), the supernatant was subjected to air blowing for 6 h at 50 °C to eliminate volatile compounds. Finally, it was purified with activated charcoal (AC) for 1 h in a proportion of 4 g per 100 mL of hydrolysate to remove phenolic compounds that can inhibit cell growth (Chandel et al., 2011; López-Linares et al., 2020; Plaza et al., 2020). This sample is considered the beer bagasse hydrolysate (BBH) used as carbon source.

2.2. Spent brewer's yeast hydrolysis

The spent brewer's yeast has also been kindly donated by La Cibeles S.L. The yeast was mixed with water at a ratio of 10 % w/v and adjusted to pH 6.8 by addition of NaOH. Enzymatic hydrolysis was performed with Alcalase® in a proportion of 2 g kg^{-1} dry brewer's yeast (brewer's yeast had a moisture of 43 %) for 12 h at 60 °C and 200 rpm (Jiang et al., 2010). The sample was centrifuged for 15 min at 4 °C and 8,000 rpm and the supernatant collected, the spent brewer's yeast hydrolysate (SBYH), was used as nitrogen source.

2.3. Microorganism reactivation, adaptation and inoculum

The microorganism used was *A. succinogenes* DSM 22257 (German Collection of Microorganisms and Cell Cultures GmbH). For its reactivation, cells maintained at -80 ºC in a Tryptic Soy Broth (TSB)/glycerol 50 % v v^{-1} mixture were thawed and incubated at 37 ºC and 200 rpm for 24 h in bottles with TSB medium. TSB composition

was (in grams per liter): 17 Tryptone, 3 Soytone, 2.5 Glucose, 5 NaCl, 2.5 K_2HPO_4. The air in the bottles had been previously displaced by N_2 bubbling.

To achieve high yields and a good reproducibility, the cells were reactivated and adapted to the carbon source (Escanciano et al., 2022a). For this, successive cell growths were carried out under anaerobic conditions at 37 ºC, 200 rpm in bottles with increasing concentrations of xylose (until reaching 20 g L^{-1}) and 60 mL of production medium, whose composition was (in grams per liter): 3 K_2HPO_4, 0.43 $MgCl_2.6H_2O$, 0.2 $CaCl_2$, 1 NaCl, 10 yeast extract (YE). In addition, both $NaHCO_3$ and the amount of xylose in the medium were added and the pH was adjusted to 6.8. In the event that a fermentation with BBH was subsequently carried out, an adaptation was performed by means of consecutive growths in bottles, under the conditions indicated for the adaptation stage to xylose, with increasing percentages of the hydrolysate until it completely replaced the carbon source. This stage was not necessary to carry out fermentations with SBYH as a nitrogen source. After the adaptations, the last growth of the microorganism was carried out in the inoculum stage. This growth was done under the same operating conditions and with the same composition as in the last step of adaptation to the carbon source, starting from an initial biomass concentration of 0.05 g L^{-1}.

2.4. Bottle production under different dilution factors (DF) of SBYH

In order to determine the best SBYH concentration, a series of experiments was carried out in bottles at 37 ºC, 200 rpm with 20 g L^{-1} of commercial xylose and the previously described production medium but substituting the commercial YE for SBYH. Starting with 0.05 g L^{-1} of biomass, fermentations were carried out with culture media whose

composition was 50 % SBYH, DF 2, up to media whose proportion of SBYH was 8.3 % (DF 12). As a control, a production of bottles with 10 g L^{-1} YE as a nitrogen source was also carried out.

2.5. Succinic acid bioproduction in a batch reactor.

The production of succinic acid in a batch reactor was performed in a 2-L stirred tank BIOSTAT B-Plus (Sartorius AG, Germany). The operating conditions were: 37 ºC, 300 rpm, pH 6.8 (5M NaOH), CO_2 flow rate of 0.1 vvm and a working volume of 1 L. The previously described production medium was used with 20 g L^{-1} of xylose but without addition of $NaHCO_3$. In the pertinent experiments, commercial xylose was substituted by BBH in sufficient quantity to reach 20 g L^{-1} of xylose. Experiments were also carried out in which YE was substituted for SBYH with the DF previously determined to be optimal. The fermentations began after the inoculation of 0.05 g L^{-1} of biomass.

2.6. Analytical methods

Biomass concentration was determined by UV-vis spectrophotometry (Shimadzu UV-Vis spectrophotometer UV-1603, Japan) at 600 nm.

Substrate and products concentration were quantified through an Agilent Technologies 100 series equipment, USA, by high-performance liquid chromatography (HPLC). For the sugars analysis, a BP-800 Pb column (8 %, 300 x 7.8 mm, Benson) was used. For the determination of the acids concentration, a BP-800 H column (8 %, 300 x 7.8 mm, Benson) was chosen. Both columns worked at 80 ºC with a H_2SO_4 5 mM solution at a flow rate of 0.5 mL min^{-1}. The refraction index detector temperature was 55 ºC.

2.7. Theory/Calculation

In order to compare the results obtained, various fermentation parameters such as titer (C_{SA}, g L^{-1}), yield ($Y_{SA,}$ g g^{-1}), productivity (P_{SA}, g L^{-1} h^{-1}) and selectivity (S_{SA}, g g^{-1}) are used throughout this work, according to the following equations:

$$Y_{SA} = \frac{C_{SA}}{C_{S_0}} \quad (1)$$

$$Y_{SA,Scons} = \frac{C_{SA}}{C_{S_{cons}}} \quad (2)$$

$$P_{SA} = \frac{C_{SA}}{t} \quad (3)$$

$$S_{SA} = \frac{C_{SA}}{C_{SA} + C_{FA} + C_{AA}} \quad (4)$$

Where C_i is the concentration of compound I, SA is succinic acid, S0 is substrate at time zero, S_{cons} is the consumed substrate, FA is formic acid, AA is acetic acid and t is time.

Likewise, a simple kinetic model, of the unstructured-non-segregated type, previously developed by the research group, was fitted to relevant data (Escanciano et al., 2023). The aforementioned model is made up of the equations included in equations (5) to (14), with equations (5) to (7) corresponding to the simplified reaction scheme, in which, for its application in this work, all the consumed sugars have been lumped in a single compound, called S, and lumping has also been applied to the two acids (formic and acetic) that are obtained as by-products, calling the compound BP. The model is made up of three reactions, whose kinetic equations are collected in expressions (8) to (10). Finally, equations (11) to (14) collect the set of differential equations used in the statistical adjustment of the model to the experimental data.

Reaction network:

$$Y_{S/X}S \xrightarrow{r_1} X \quad (5)$$

$$Y_{S/P1}S \xrightarrow{r_2} SA + Y_{S/BP} \cdot BP \quad (6)$$

$$Y_{S/P2}S \xrightarrow{r_3} BP \quad (7)$$

Reaction rates

$$r_1 = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_{Xm}}\right) \quad (8)$$

$$r_2 = k_{P1} \cdot C_S \cdot C_X \quad (9)$$

$$r_3 = k_{P2} \cdot C_S \cdot C_X \quad (10)$$

Production and consumption rates

$$R_S = \frac{dC_S}{dt} = -Y_{S/X} \cdot r_1 - Y_{S/P1} \cdot r_2 - Y_{S/P2} \cdot r_3 \quad (11)$$

$$R_{SA} = \frac{dC_P}{dt} = r_2 \quad (12)$$

$$R_X = \frac{dC_X}{dt} = r_1 \quad (13)$$

$$R_{BP} = \frac{dC_{BP}}{dt} = Y_{S/BP} \cdot r_2 + r_3 \quad (14)$$

In these equations r_i (g L^{-1} h^{-1}) is the reaction rate of reaction "i", R_j (g L^{-1} h^{-1}) is the consumption or formation rate of compound "j", μ (h^{-1}) is the specific biomass growth rate, C_{Xm} (g L^{-1}) is the maximum biomass concentration, k_{P1}Y k_{P2} (L g^{-1} h^{-1}) are the kinetic constants and $Y_{S/X}$, $Y_{S/P1}$, $Y_{S/BP}$ and $Y_{S/P2}$ (g g^{-1}) are the macroscopic yields.

3. Results

The experimentation carried out in the present work includes several aspects: the hydrolysis of BBH and its characterization (considering different hydrolysis and/or

detoxification techniques) for using it as a carbon source in the production of succinic acid with *A. succinogenes* DSM 22257; the hydrolysis of SBYH and the determination of the best concentration of this hydrolysate as a nitrogen source in the process; the study of the feasibility of using each of the beer wastes to replace the commercial carbon (xylose) and nitrogen (YE) sources separately and, finally, the use of both wastes as carbon (BBH) and nitrogen (SBYH) sources in the bioprocess. Furthermore, with the aim of providing information for techno-economic studies of the integral production process of succinic acid from beer wastes, a kinetic model developed by the research group was applied to check its capacity to describe the process based on beer solid wastes.

3.1. Characterization of the beer bagasse hydrolysate (BBH)

In the hydrolysis of BBH, after the action of α-amylase that allowed the hydrolysis of the α-1,4 glycosidic bonds in amylose and amylopectin, so that the starch was rapidly broken down into soluble dextrins and oligosaccharides, it was possible to apply a cocktail of endo-β-glucanases that hydrolysed (1,3) or (1,4) linkages in β-D-glucans and xylanases that hydrolysed (1,4)-β-D-xylosidic linkages in xylans, Endoproteases hydrolysed internal peptide bonds, and glucoamylases broke dextrins down to simple sugars. Finally, thanks to a mixture of endo- and exo- glucanases, the cleavage of the cellulose polymer into smaller sugars and oligomeric polysaccharides was achieved, obtaining a hydrolysate whose composition is shown in Table 2.

In the detoxified and volatile-free hydrolysate, a final sugar concentration of 124.6 g L^{-1} was obtained, of which the majority was xylose. The preponderance of this pentose is in agreement with the results obtained by other authors (Rojas-Pérez et al., 2022). The rest

of the sugars were released in a much lower proportion, in fact, the sum of all of them barely accounts for 16% of the total sugar composition.

3.2. Determination of the dilution factor of the spent brewer's yeast hydrolysate (SBYH)

As previously mentioned, a study was carried out to determine the most appropriate concentration of SBYH for the production of succinic acid had compared the production of succinic acid from SBYH and yeast extract in quantities such that total nitrogen concentrations coincided, although the total amounts of amino acids differed (Jiang et al., 2010). Therefore, considering the bioavailability of the nitrogen source more important than the amount itself, five dilutions of the aforementioned residue were performed and subsequently used in fermentation experiments as a source of nitrogen, with the aim of determining the adequate amount of this hydrolysate that allows to carry out productions of succinic acid with the same performance as with commercial yeast extract. The results are shown in Table 3, in which they are compared to a control experiment carried out using 10 g L^{-1} YE as a nitrogen source in terms of titer, yield, selectivity and productivity after 24 h fermentation. As it can be seen the results obtained for DF6 are the closest to those ones obtained when using the commercial YE.

The decrease in the dilution factor of SBYH leads to a reduction in selectivities. Despite this, the highest values of yield and productivity were not reached with the lowest dilution factor (DF 12), but with the next one that was studied (DF 6). When using a DF 12, the yield of succinic acid in relation to the xylose consumed was very similar to that corresponding to the fermentation with DF 6. However, the differences between the yields of succinic acid depending on the amount of initial xylose are much greater (16

% for DF 12 and 23 % for DF 6), which seems to indicate an insufficient quantity of nutrients with a DF 12. The yield and productivity values with DF 6, DF 4 and DF 3 are very similar, but, as with DF 6 the amount of by-products generated is lower and also a smaller amount of SBYH is used. Therefore, it was considered that the best option was to perform fermentation with this amount of hydrolysate.

3.3. Succinic acid bioproduction with xylose or BBH as a carbon source

Keeping yeast extract as nitrogen source at a concentration of 10 g L^{-1}, two experiments were performed to study the effect of carbon source: xylose (pure reagent) and beer bagasse hydrolysate (whose main sugar is xylose, as shown in Table 2). Figure 2 shows the results obtained in batch experiments. It is observed that the growth when beer bagasse is used is somewhat faster and a higher biomass concentration is reached (Figure 2C). It should be noted that the hydrolyzed bagasse does not only have xylose as a sugar in its composition, so in Figure 2B the consumption of the rest of the sugars can be observed: maltose, glucose, arabinose, mannose and fructose are completely consumed around 17 h fermentation; however, the microorganism is not able to metabolize galactose. As for xylose consumption (Figure 2B), the consumption of xylose as a pure reagent is somewhat faster, but it is practically exhausted around 27 h for both xylose sources (pure and BBH). Finally, the succinic acid production achieved (see Figure 2A) is higher when BBH is used (13.3 g L^{-1}), although it is not much higher than that obtained with 20 g L^{-1} of pure xylose (10.6 g L^{-1}), as shown in Table 1. As for the two by-products obtained in the process (formic acid and acetic acid), no significant difference is observed between the use of pure xylose or BBH. Regarding productivity (see Table 1), when using BBH its value suffers a slight increase compared to that obtained with pure xylose (0.41 versus 0.38 g L^{-1} h^{-1}). It is concluded that the use of

BBH favors the selectivity of succinic acid production, being 57% with this residue compared to 50% in the case of using commercial xylose.

As can be seen in Table 1, the yield of the operation with BBH (61 %) not only exceeds that achieved in the fermentation with pure xylose (53 %), but also the values reached in other works in which they operated in batch using this pentose as a substrate and YE as nitrogen source, such as (Almqvist et al., 2016), who managed to obtain yields of 42 % also using *A. succinogenes* as a biocatalyst. (Oreoluwa Jokodola et al., 2022b) reached significantly lower yields (27 %) but achieved one of the highest batch production rates from pure xylose, with a productivity of 0.51 g L^{-1} h^{-1}. (Ferone et al., 2017) simulated the behavior of a fermentation from lignocellulosic waste carrying out a batch-type operation from a mixture of sugars representative of what could be obtained after hydrolysis of this type of biomass, obtaining yields very similar to those of this work with pure xylose (55 %). In recent years, the volume of publications focused on the production of succinic acid from residues has grown rapidly, although the use of those rich in xylose is still a minority. Among the latest studies, it is worth highlighting that of (Oreoluwa Jokodola et al., 2022b), who managed to produce 33.7 g L^{-1} of succinic acid from olive pits reaching exactly the same performance as when they used pure xylose, or the work carried out by (Lee et al., 2022) who, despite the fact that batch operations do not usually allow high reaction rates, produced 0.79 g L^{-1} h^{-1} of succinic acid from Napier grass. With a fed-batch type operation, (Filippi et al., 2021) managed to reach the same productivity value that the latest authors from grape stalks and pomace, but with a higher yield (67 %). Thanks to a repeated batch operation with immobilized cells in agar, (Corona-González et al., 2016) were able to considerably increase the production speed (1.32 g L^{-1} h^{-1}), although with a yield that was limited to 39 %.

3.4. Succinic acid bioproduction with YE or SBYH as nitrogen source

To compare the influence of YE (10 g L^{-1}) or SBYH (DF 6) on the process, two experiments were performed using pure xylose at a concentration of 20 g L^{-1} as carbon source. The results of the evolution over time of the different compounds present in the processes (xylose, biomass, and succinic, acetic and formic acids) are shown in Figure 2. In the aforementioned Figure it can be observed that, due to the fact that the consumption of xylose when using YE is faster (Figure 2A), the process in the experiment carried out with the aforementioned nitrogen source ends after 30 hours. However, when diluted SBYH is used, the speed of the whole process (substrate consumption and acid production) slows down. It should be noted that the growth of the microorganism (Figure 2C) is not affected by the nitrogen source used, being carried out at the same rate in the two experiments carried out.

With respect to acid production (Figure 2B), it is noteworthy that the use of the residue as a source of nitrogen significantly reduces the production of by-products (reaching a selectivity of 68 %, which implies an increase of 36 % compared to the equivalent process with YE), while, although its use slows down the rate of succinic acid production, the titer and yield obtained are higher (12.8 g L^{-1}; 0.61 g g^{-1}) than that corresponding to the use of YE (10.6 g L^{-1}; 0.53 g g^{-1}), as can be observed in Table 1. However, the productivity with SBYH (0.14 g L^{-1} h^{-1}) turns out to be half that with YE (0.38 g L^{-1} h^{-1}).

It can be appreciated that the replacement of the nitrogen source has hardly been studied in depth, despite being one of the greatest limitations in these types of processes due to its high costs. Table 1 shows the results achieved in two succinic acid production

processes with replacement of the nitrogen source by food waste, such as the previously mentioned work by (Jiang et al., 2010), who managed to produce succinic in a batch type operation from glucose and SBYH supplemented with biotin with a yield and productivity of 68 g g^{-1} and 0.63 g L^{-1} h^{-1}, respectively (it should be mentioned that in the productions in which glucose is used as a carbon source, as a rule general, higher values in these type of parameters are obtained (Bradfield and Nicol, 2016; Ercole et al., 2021; Ferone et al., 2017; Kim et al., 2021; Luthfi et al., 2018; Salvachúa et al., 2016; Zhang et al., 2020)). In the work of (Filippi et al., 2022), they opted to carry out a fed-batch type operation that would allow optimizing the performance of succinic acid production using wine lees as a nitrogen source, producing 37.2 g L^{-1} of succinic acid in 47 h. In a previous study by this research group, (Escanciano et al., 2022a) managed to produce succinic acid in the absence of a nitrogen source, using cells in a resting state, reaching a yield of 43 % and reducing the by-product formation dramatically compared to the same operation carried out with cells in a growing state.

3.5. Succinic acid bioproduction with BBH and SBYH as carbon and nitrogen sources

Once the possibility of using BBH and SBYH as carbon and nitrogen sources, respectively, in the production of succinic acid was proven, an experiment was carried out using both residues as substitutes for commercial xylose and YE. Figure 3 shows the time course of the results obtained in this experiment for biomass growth, acid production (Figure 3A) and consumption of sugars present in the BBH (Figure 3B). As can be seen in the Figure, the succinic acid production process using both residues is viable, obtaining a concentration of succinic acid of 15.6 g L^{-1}, higher than those obtained in the rest of the experiments carried out (see Table 1). Likewise, as observed

in the experiment carried out with BBH and YE, the productivity is reduced to approximately half of that observed when pure xylose is used; however, the yield to the target acid obtained (0.77 g g^{-1}) is the highest of all the experiments carried out, including the experiment carried out without residues (0.53 g g^{-1}), as shown in Table 1. It is true that it must be taken into consideration that the BBH residue presents a total concentration of sugars higher than the 20 g L^{-1} of xylose used as control. Considering that high cost succinic acid purification techniques are one of the main disadvantages of the bioproduction process (Salma et al., 2021), it is worth noting that the simultaneous use of the brewery residues as carbon and nitrogen source, instead of commercial xylose and YE, implies doubling the selectivity of the process, reaching a value of 76 %.

As mentioned, the experimental data were finally adjusted to a previously developed kinetic model. As has been commented in the Theory/Calculations section, the equations have been used to fit the model to the experimental data collected in Figure 4, in which the evolution of lumped sugars is represented as points, as a single compound, succinic acid, biomass and by-products. We can appreciate in the aforementioned figure that the fit of the model to the data is very good (represented as lines in Figure 4). Likewise, Table 4 shows both the values of the statistical fit parameters obtained (which reinforce the goodness of fit), as well as those corresponding to the model parameters, used for the simulation collected in the form of lines in Figure 4.

4. Conclusions

Bioproduction of succinic acid using wastes from a brewery in an integral manner, as a circular economy concept is demonstrated. The substitution of the glucose by BBH led to higher yields and productivities. When commercial YE was replaced by SBYH,

productivity decreased but both yield and selectivity. Succinic acid production from the secondary raw materials simultaneously obtained the highest yield (77 %) and a productivity of 0.15 g L^{-1} h^{-1}. Finally, a simple and accurate kinetic model could be successfully applied in this last run which will facilitate the future performance of techno-economic analyses and scaling of the process.

Author contributions

Itziar A. Escanciano: Methodology, Software, Investigation, Data curation, Writing-original draft preparation, Visualization. **Ángeles Blanco**: Conceptualization, Validation, Resources, Writing—review and editing, Visualization, Supervision, Project administration, Funding acquisition. **Victoria E. Santos**: Conceptualization, Software, Validation, Formal analysis, Resources, Data curation, Writing—review and editing, Visualization, Supervision, Funding acquisition. **Miguel Ladero**: Conceptualization, Validation, Formal analysis, Resources, Data curation, Writing—review and editing, Visualization, Supervision, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure Captions

Figure 1. Time course of (A) succinic, acetic and formic acids, (B) sugars and (C) biomass in runs using commercial xylose (open symbols) and BBH (close symbols) as carbon source.

Figure 2. Time course of (A) succinic, acetic and formic acids, (B) xylose and (C) biomass in runs with commercial YE (open symbols) and SBYH (closed symbols) as nitrogen source.

Figure 3. Time course of succinic acid, by-products and biomass (A) and sugars (B) when using BBH as carbon source and SBYH as nitrogen source.

Figure 4. Simulation of time course of succinic acid, by-products, total sugars and biomass when using BBH as carbon source and SBYH as nitrogen source by means of a fitting of a proposed kinetic model using parameters from Table 4. Points: experimental data; lines: model predictions.

Tables

Table 1. Comparison of the bibliographic results of succinic acid production through the action of *A. succinogenes* with those corresponding to this study in a reactor using BBH or commercial xylose as carbon source and SBYH or YE as nitrogen source.

Type of operation	**Cells state**	**Carbon source**	**Nitrogen source**	**C_{SA} ($g \cdot L^{-1}$)**	**Y_{SA} ($g \cdot g^{-1}$)**	**P_{SA} ($g \cdot L^{-1} \cdot h^{-1}$)**	**Reference**
Batch	Growing, Free	Xylose	YE	3.94	0.42	0.15	(Almqvist et al., 2016)
Batch	Growing, Free	Xylose	YE	36.7	0.27	0.51	(Oreoluwa Jokodola et al., 2022a)
Batch	Growing, Free	Lignocellulosic sugars	YE	27.0	0.55	0.22	(Ferone et al., 2017)
Batch	Resting, Free	Xylose	None	8.51	0.43	0.18	(Escanciano et al., 2022a)
Continuous	Growing, Immobilized	Xylose	YE, Corn steep liquor	29.4	0.68	3.4	(Bradfield and Nicol, 2016)
Fed-batch	Growing, Free	Olive pits	YE	33.7	0.27	0.50	(Oreoluwa Jokodola et al., 2022a)
Batch	Growing, Free	Napier grass	YE	17.54	0.58	0.79	(Lee et al., 2022)
Repeated batch	Growing, Immobilized	Tequilana agave bagasse	YE	33.6	0.39	1.32	(Corona-González et al., 2016)
Batch	Growing, Free	Glucose	SBYH	47.6	0.68	0.63	(Jiang et al., 2010)
Fed-batch	Growing, Free	Grape stalks and pomace	YE	40.2	0.67	0.79	(Filippi et al., 2021)
Fed-batch	Growing, Free	Grape stalks and pomace	Wine lees	37.2	0.64	0.79	(Filippi et al., 2022)
Batch	Growing, Free	Xylose	YE	10.6	0.53	0.38	This study
Batch	Growing, Free	BBH	YE	13.3	0.61	0.41	This study
Batch	Growing, Free	Xylose	SBYH	12.8	0.61	0.14	This study
Batch	Growing, Free	BBH	SBYH	15.6	0.77	0.15	This study

Table 2. Sugar concentration of beer bagasse after hydrolysis, after removal of volatiles by aeration and after the addition of a purification step with activated carbon (AC)

Compound	**Concentration (g L^{-1})**
Sucrose (SAC)	0.00
Maltose (MALT)	1.57
Glucose (GLUC)	0.57
Xylose (XYL)	105
Galactose (GALACT)	11.8
Arabinose (ARAB)	0.19
Mannose (MANN)	3.87
Fructose (FRUCT)	1.56

Table 3. Succinic acid concentration, yield, selectivity and productivity after 24 h of fermentation with SBYH at different dilution factor (DF) and commercial YE as nitrogen sources.

Fermentation Parameters	**SBYH**					**YE**
	DF 12	DF 6	DF 4.5	DF 3	DF 2	
C_{SA} (g L^{-1})	3.45	4.41	4.17	3.98	3.69	4.39
S_{SA} (g g^{-1})	0.69	0.67	0.61	0.40	0.36	0.52
Y_{SA} (g g^{-1})	0.16	0.23	0.21	0.21	0.18	0.22
$Y_{SA/S.cons}$ (g g^{-1})	0.58	0.60	0.54	0.38	0.33	0.60
P_{SA} (24 h) (g L^{-1} h^{-1})	0.14	0.18	0.17	0.17	0.15	0.18

Table 4. Kinetic and statistical parameter values calculated by fitting the kinetic model to experimental data of succinic acid production with BBH as carbon source and SBYH as nitrogen source.

C_{Xm} (g L^{-1})	3.20	±	0.07	**F_{95}**	8,016
kp_1 10^{-3} (L h g^{-1})	3.06	±	0.21	**RMSE**	0.36
kp_2 10^{-2} (L h g^{-1})	8.52	±	0.07	**SSR**	6.52
μ (h^{-1})	0.41	±	0.01	**VE (%)**	99.4
$Y_{S/P1}$ (g g^{-1})	0.39	±	0.02		
$Y_{S/P2}$ (g g^{-1})	1.99	±	0.21		
$Y_{S/BP}$ (g g^{-1})	0.55	±	0.04		
$Y_{S/X}$ (g g^{-1})	0.73	±	0.03		

Figures

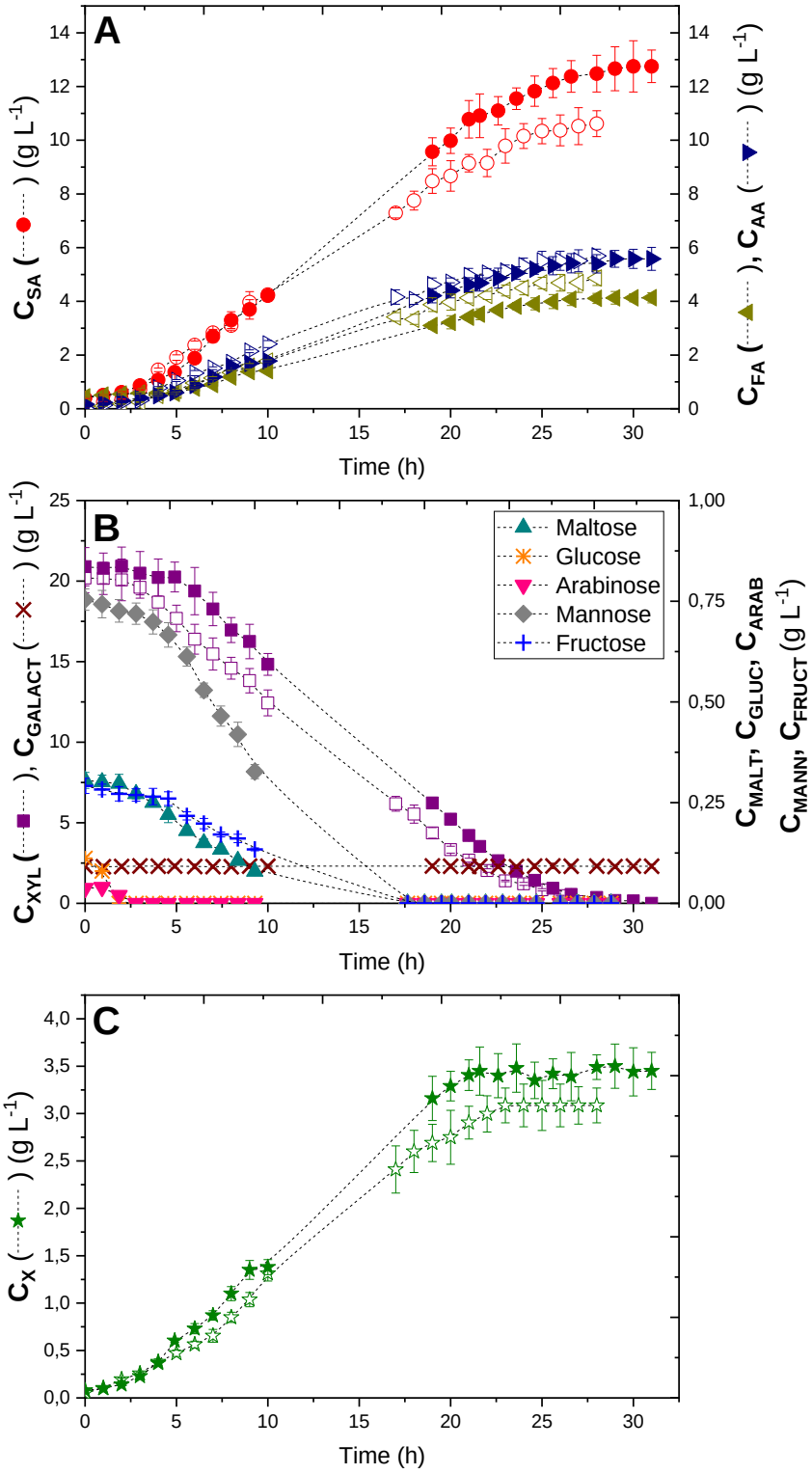

Figure 1.

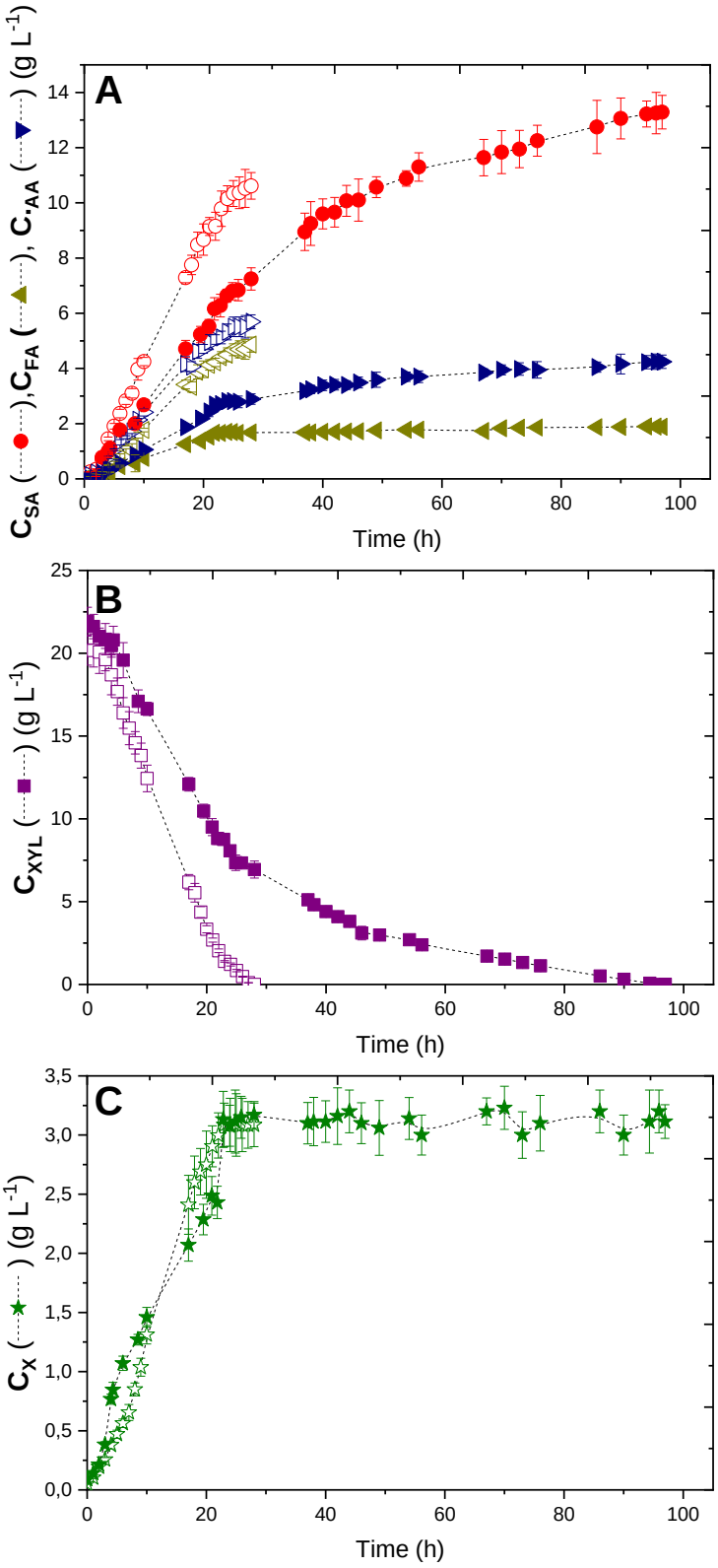

Figure 2.

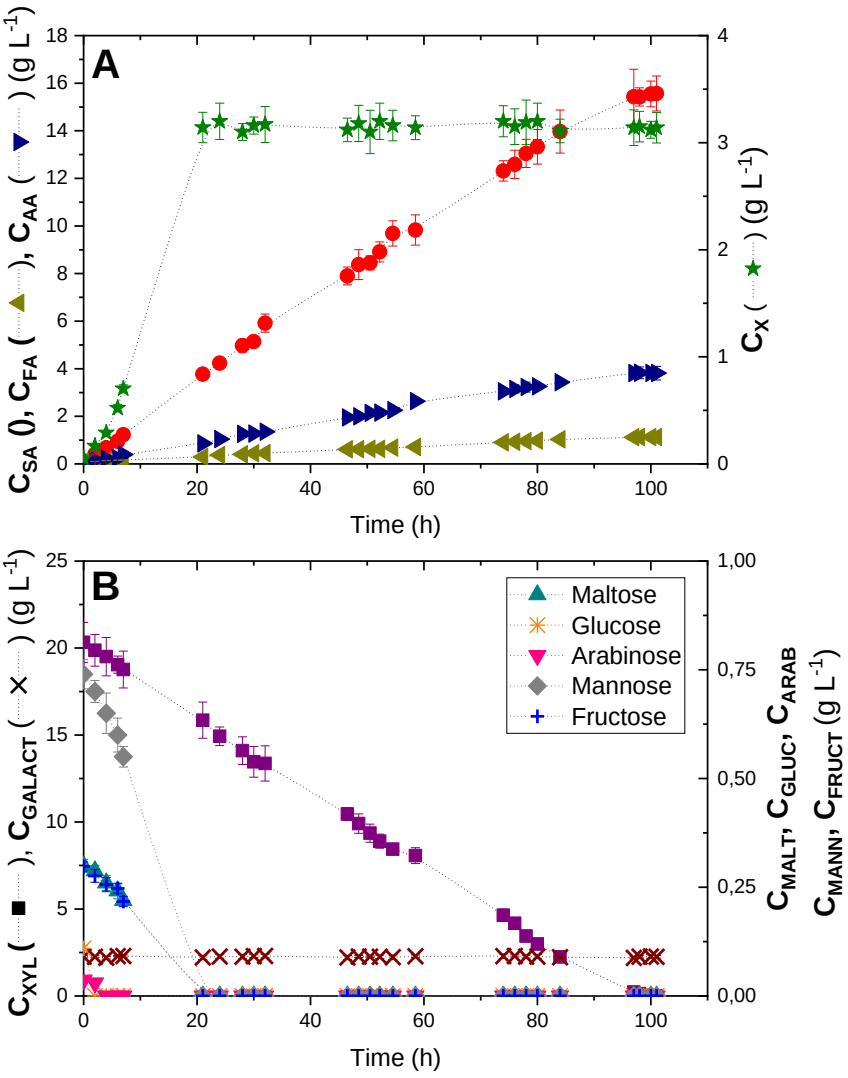
A
B
Time (h)
Time (h)
Maltose
Glucose
Arabinose
Mannose
Fructose

Figure 3

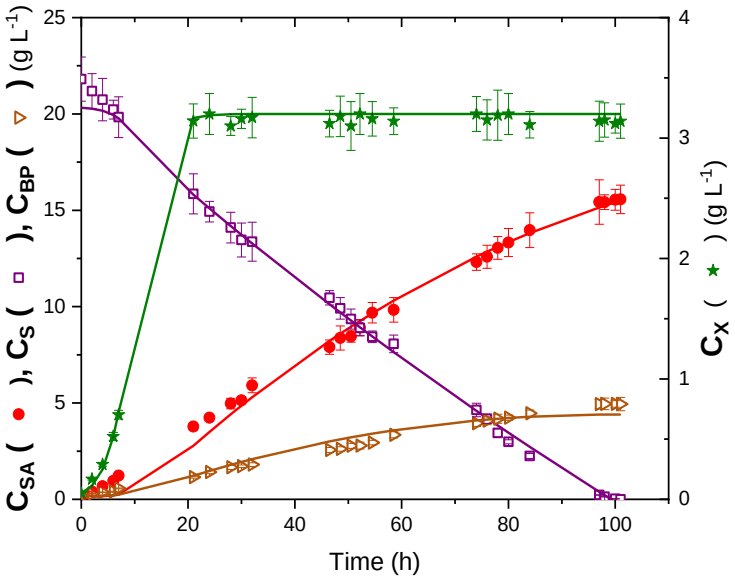

Figure 4

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

8.7 PUBLICACIÓN 7

Autores: Itziar A. Escanciano, Miguel Ladero, Ángeles Blanco y Victoria E. Santos

Título: Succinic acid production by *Actinobacillus succinogenes* using acid and enzymatic hydrolysates of potato and beer wastes and repeated batch operation

Revista: *Chemical Engineering Journal* (en revisión)

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Categoría: Ingeniería Química, Ingeniería Medioambiental

Resumen:

La sustitución de recursos fósiles por biomasa es uno de los puntos clave para el desarrollo de la bioeconomía. En este trabajo se utilizaron residuos de alimentos como materia prima para la bioproducción de ácido succínico, un químico de plataforma de gran versatilidad e importancia en las biorrefinerías. La biomasa utilizada fueron patatas desechadas y levadura de cerveza gastada, que sirvieron como fuente de carbono y nitrógeno, respectivamente. Se utilizó como biocatalizador la bacteria anaeróbica *Actinobacillus succinogenes*. Para mejorar el rendimiento de este proceso, se realizaron fermentaciones tipo *repeated batch*, comparando la evolución del proceso utilizando hidrolizado de desechos de patatas o glucosa pura e hidrolizado de levadura de cerveza gastada o extracto de levadura comercial. Los experimentos en los que se sustituyeron completamente las fuentes de carbono y nitrógeno por residuos, se produjeron 101 g L^{-1} de ácido succínico, con un rendimiento de 0,84 g g^{-1} y una productividad de 1,02 g L^{-1} h^{-1}. Finalmente, se aplicó con éxito un modelo cinético previamente desarrollado, obteniendo los valores de los parámetros correspondientes al uso estos residuos.

Chemical Engineering Journal

Succinic acid production by Actinobacillus succinogenes using acid and enzymatic hydrolysates of potato and beer wastes and repeated batch operation

--Manuscript Draft--

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Article Type:	Research Paper
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Keywords:	succinic acid; spent brewer's yeast; potato waste; repeated batch; fermentation; kinetic model
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	Victoria Santos, Ph.D.
Abstract:	The replacement of fossil resources by biomass is one of the key points for the development of bioeconomy. Here we have focused on the bioproduction of succinic acid, a biorefinery platform of great versatility and importance. In this study, the anaerobic bacterium Actinobacillus succinogenes was used as the biocatalyst and discarded potatoes and spent brewer's yeast hydrolysates served as carbon and nitrogen sources, respectively. The use of food residues is key to reduce OPEX in this type of processes. Furthermore, to analyze and improve the process performance, repeated batch fermentations were carried out in several conditions: with potato waste hydrolysate or pure glucose as carbon sources and spent brewer's yeast hydrolysate or commercial yeast extract as nitrogen sources. Very promising results were obtained with the residue preparations, 35.8 g L-1 of succinic acid were produced, with a yield of 0.84 g g-1 and an average productivity of 1.19 g L-1 h -1, increasing the selectivity towards the target acid in comparison to pure glucose and yeast extract. Finally, a previously developed unstructured non-segregated global kinetic model is successfully applied, obtaining the parameters values in all conditions.

Prof. Dr. Soryong Ryan Chae

Editor-in-Chief Chemical Engineering Journal

Madrid, 22th March 2023

Dear Prof. Dr. Soryong Ryan Chae

Attached to this letter you may find files containing the manuscript, figures and tables, graphical abstract and highlights of a research paper entitled "Succinic acid production by *Actinobacillus succinogenes* using acid and enzymatic hydrolysates of potato and beer wastes and repeated batch operation" that we wish to publish in Chemical Engineering Journal. The authors are Itziar A. Escanciano, Miguel Ladero, Angeles Blanco and Victoria E. Santos. All authors have agreed to publish this review in Chemical Engineering Journal, being this the first time this manuscript is submitted for publication.

In this paper we have studied in depth the bioproduction of succinic acid, a biorefinery platform of great versatility and importance, using the anaerobic bacterium *Actinobacillus succinogenes* and discarded potatoes and spent brewer's yeast hydrolysates as carbon and nitrogen sources, respectively. As observed in several technoeconomic studies for biologically-derived organic acids, such as succinic, fumaric, and lactic acids, the use of food residues is key to reduce OPEX in this type of processes. Complex LCA analyses point out the importance of C4 acids, such as succinic, malic and fumaric acid, as CO_2 sinks where this harmful compound is put to good use by biological strategies. Herein, we have analyzed and improved the process performance by using repeated batch fermentations in diverse conditions: with potato waste hydrolysate or pure glucose as carbon sources and spent brewer's yeast hydrolysate or commercial yeast extract as nitrogen sources. Very promising results were obtained with the residue preparations, 35.8 g L^{-1} of succinic acid were produced, with a yield of 0.84 g g^{-1} and an average productivity of 1.19 g L^{-1} h^{-1}, increasing the selectivity towards the target acid in comparison to pure glucose and yeast extract. Finally, a previously developed unstructured non-segregated global kinetic model is successfully applied, obtaining the parameters values in all conditions. The comparison of production kinetic constant ratios sheds a light on the shift towards the succinic acid production derived from the usage of appropriate food waste hydrolysates, as those studied in this research.

Hoping the contents of this manuscript fulfils the standards and expectations of Chemical Engineering Journal, I remain sincerely yours.

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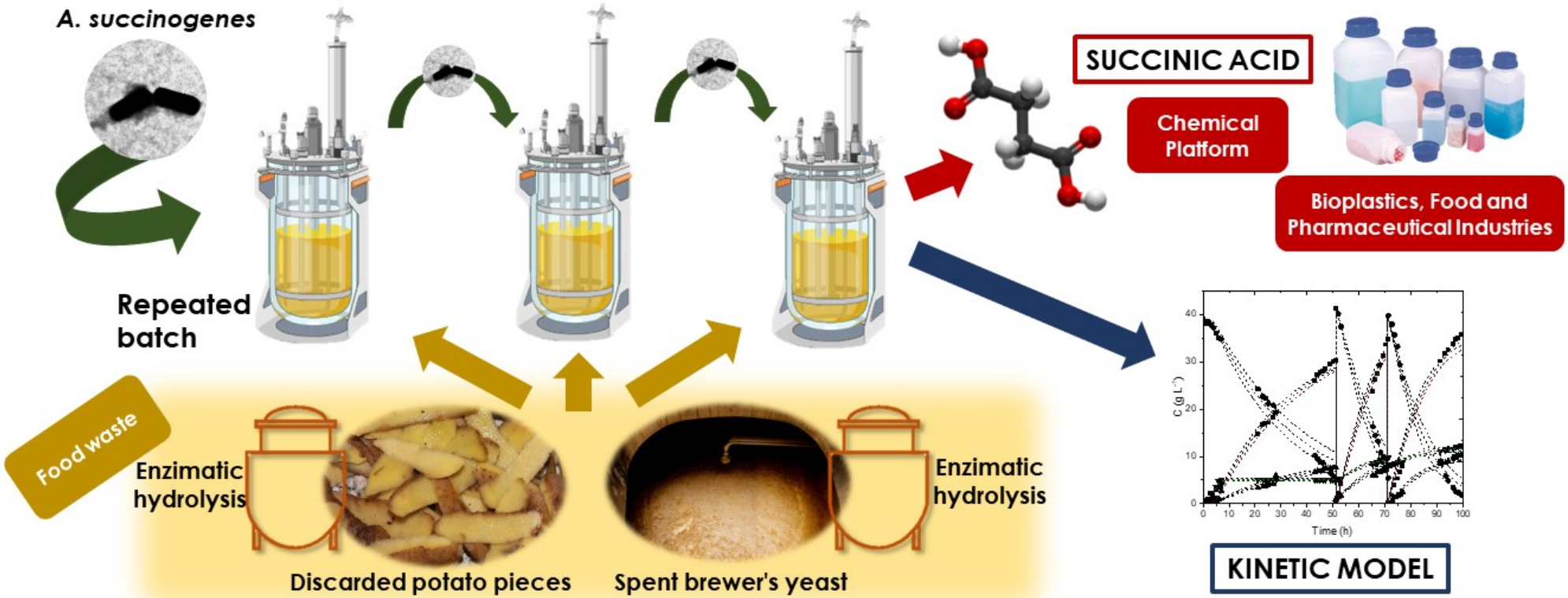
A. succinogenes
Repeated batch
SUCCINIC ACID
Chemical Platform
Bioplastics, Food and Pharmaceutical Industries
Food waste
Enzimatic hydrolysis
Enzimatic hydrolysis
Discarded potato pieces
Spent brewer's yeast
Time (h)
KINETIC MODEL

Highlights

- High yield bioproduction of succinic acid in repeated batch using food wastes
- Potato waste and spent brewer’s yeast hydrolysates as carbon and nitrogen source
- Higher productivities in the last stages of repeated batch fermentations: activation
- Higher yields with residues than with commercial glucose and yeast extract
- An unstructured non-segregated kinetic model is valid for all runs.

Succinic acid production by *Actinobacillus succinogenes* using acid and enzymatic hydrolysates of potato and beer wastes and repeated batch operation

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Abstract

The replacement of fossil resources by biomass is one of the key points for the development of bioeconomy. Here we have focused on the bioproduction of succinic acid, a biorefinery platform of great versatility and importance. In this study, the anaerobic bacterium *Actinobacillus succinogenes* was used as the biocatalyst and discarded potatoes and spent brewer's yeast hydrolysates served as carbon and nitrogen sources, respectively. The use of food residues is key to reduce OPEX in this type of processes. Furthermore, to analyze and improve the process performance, repeated batch fermentations were carried out in several conditions: with potato waste hydrolysate or pure glucose as carbon sources and spent brewer's yeast hydrolysate or commercial yeast extract as nitrogen sources. Very promising results were obtained with the residue preparations, 35.8 g L^{-1} of succinic acid were produced, with a yield of 0.84 g g^{-1} and an average productivity of 1.19 g L^{-1} h^{-1}, increasing the selectivity towards the target acid in comparison to pure glucose and yeast extract. Finally, a previously developed unstructured non-segregated global kinetic model is successfully applied, obtaining the parameters values in all conditions.

Keywords: succinic acid; spent brewer's yeast; potato waste; repeated batch; fermentation; kinetic model.

1. Introduction

The high growth rate of the world population poses a great challenge for the fulfillment of the Sustainable Development Goals (SDG) [1], resulting in great food uncertainty and environmental problems such as pollution, global warming and the depletion of natural resources [2]. Oil demand is expected to increase from 99.4 barrels/day in 2022 to 104.1 barrels/day in 2026 [3], with Asia Pacific accounting for 90% of the increase in these amounts [4]. The development of technologies for the recovery of biomass is one of the

most robust alternatives for reducing global dependence on fossil fuels, since it is an economical and sustainable source of both energy and chemical products.

However, to develop a bioeconomy based on biorefineries capable of operating competitively with those that use fossil resources, it is essential to use sustainable biomass that, apart from not serving as a source of food, does not imply an increase in arable land for its production. According to the Food and Agriculture Organization (FAO), around 1,300 million tons of food are wasted annually, for the cultivation of which around 28% of the available agricultural area is used [5,6]. Furthermore, the amount of food waste per year is predicted to increase by one third by 2030, in other words, 66 tons of food wasted per second [7]. For this reason, among other research areas, the study of the generation of added value compounds through fermentation processes is also gaining great interest in the use of food waste as carbon and nitrogen sources.

According to the United States Department Of Energy (US DOE), succinic acid (SA, 1,2-ethanedicarboxylic acid, $C_4H_6O_4$) is a key compound for the development of biorefineries within the so-called top 12 bio-based platform chemicals [8–10]. In 2022, the global market of this product was around 160 million $ with an estimated annual increment of 6.5% for the next 10 years. Thus, the market is expected to be higher than 300 million US$ in 2032. In addition, increasing health awareness and environmental concerns by governments around the world are factors that are strongly driving the demand for bio-based succinic acid, which will further push the market for this acid [11].

Succinic acid has many applications: it has been widely used in the food industry as an acidulant, flavoring and sweetener [8], as well as in the pharmaceutical industry and for the production of green solvents, detergents, corrosion inhibitors, surfactants, solvents and chelating agents [12,13]. Thanks to its great reactivity and versatility, succinic acid can be converted to other compounds such as succinimide and maleic anhydride. Another of its most promising uses is the manufacture of biodegradable polymers, via the prior conversion of this acid into dimethyl/diethyl succinate [13–16].

To date, among the succinic acid-producing microorganisms, bacteria isolated from the rumen of cattle, such as *Actinobacillus succinogenes*, are considered to have the greatest potential, due to their ability to naturally generate C4 dicarboxylic acids during pregastric digestion of polysaccharides [17–20]. These microorganisms produce bio-succinic acid

through the so-called tricarboxylic acid (TCA) cycle, requiring CO_2 for the activation of the oxaloacetic acid production route from PEP (PhosphoEnol Pyruvate) [19,21].

The literature related to the production of bio-based succinic acid is very extensive and of great variety. Researchers have studied different configurations and types of reactors, managing to considerably increase yields with repeated batch operations with support and fed-batch [22–26], in addition, thanks to continuous operations with biofilm formation, in some cases, it has been possible to improve drastically the values of productivity [26,27]. The yields and productivities are especially high when working with immobilized cells, operating both in repeated batch, as in the case of Cao et al. [28], who managed to increase the production of succinic acid by practically 50% compared to the batch operation. Another important set of studies has been carried out focused on the substitution of the carbon source. Results show that, in some cases, the efficiency of the process with residues is higher that the obtained with commercial sugars. For example, Jokodola et al. [23] compared the performance of the fermentation with olive pits and with pure commercial xylose, noting that the same yield of 27% was achieved in both cases. However, it should be noted that the replacement of the nitrogen source has hardly been studied, despite being one of the greatest limitations in these types of processes due to its high costs. It is worth highlighting the studies by Jiang et al. [29], who managed to produce succinic from glucose and brewer's yeast supplemented with biotin and from Filippi et al. [22], who managed to make full use of the waste from a winery, using it both as a carbon source (grape stems and pomace) and as a nitrogen source (wine lees).

Therefore, the aim of this work is to carry out a proof of concept regarding the production of succinic acid by fermentation by means of a repeated batch operation with *A. succinogenes* and using food residues as carbon and nitrogen sources simultaneously. For this purpose, a potato waste hydrolysate, rich in glucose, and a spent brewer's yeast hydrolysate, rich in proteins, will be used. The yield and productivity of this process will be compared with their equivalents when using commercial glucose as a carbon source and yeast extract as a nitrogen source. In addition, the kinetic parameters of all the experiments will be estimated using an unstructured non segregated model previously proposed, which can serve as a precedent for carrying out techno-economic analyses, process scaling or design of a control system.

2. Materials and methods

2.1. Discarded potato pieces hydrolysis

Residues of discarded potato pieces from a chips factory of ESPAFRIMA S.L (Getafe, Madrid) were used as carbon source. Potato pieces were peeled and washed. Acid hydrolysis of the mashed potato waste was carried out with 1M HCl in a 1:1 w v^{-1} potato/acid ratio at 100 ºC and 200 rpm for 1 hour. Discarded Potato Pieces Hydrolysate (DPPH) was centrifuged at 9,000 rpm for 10 minutes and the supernatant was sterilized by filtration.

2.2. Spent brewer's yeast hydrolysis

The spent beer yeast was provided by La Cibeles S.L brewery (Leganés, Madrid). First, a 10% w v^{-1} yeast/water mixture was adjusted to pH 6.8 (NaOH) and 2g kg^{-1} dry brewer's yeast of Alcalase® was added. Enzymatic hydrolysis was carried out for 12 h at 60 °C and 200 rpm. The Spent Brewer's Yeast Hydrolysate (SBYH) was centrifuged at 8,000 rpm for 15 min and the supernatant was also sterilized by filtration.

2.3. Microorganism, culture medium and fermentation procedure

The strain *A. succinogenes* DSM 22257 was supplied by the German Collection of Microorganisms and Cell Cultures GmbH. For long-term preservation, a Tryptic Soy Broth (TSB) culture was mixed with glycerol at a 50% v v^{-1} ratio and stored at -80 ºC. TSB composition (in grams per liter): 17 Tryptone, 3 Soytone, 2.5 Glucose, 5 NaCl, 2.5 K_2HPO_4.

For the reactivation of the thawed cells, an incubation was carried out at 37 ºC and 200 rpm for 24 h in bottles with 60 mL of TSB medium, whose oxygen had previously been displaced with N_2 (1 L min^{-1}, 2 min). Subsequently, the microorganism was adapted to the carbon source by means of successive growths with increasing concentrations of glucose in anaerobic bottles with Production Medium (PM) and the same concentration of $NaHCO_3$ (CO_2 source and pH control) as glucose. PM composition (in grams per liter): 3 K_2HPO_4, 0.43 $MgCl_2.6H_2O$, 0.2 $CaCl_2$, 1 NaCl, 10 yeast extract (YE). The inoculum growth was carried out at 37 ºC and 200 rpm, until reaching the exponential phase, in bottles with MP and 40 g L^{-1} of glucose and NaHCO3, injecting 5% v v^{-1} of cells adapted to the source of carbon. In the case that the reactor production was carried out with DPPH, the pure sugar was replaced by the hydrolysate in a sufficient amount to reach 40 g L^{-1} of

glucose. In addition, it was necessary to carry out an additional adaptation to the hydrolysate through successive growths with increasing percentages in the culture medium.

Repeated batch runs were carried out in a 2 L stirred tank bioreactor (STBR) BIOSTAT B-Plus (Sartorius AG, Germany). The operating conditions were 37 ºC and 300 rpm, while pH was controlled at 6.8 using a 5M NaOH solution and CO_2 was sparged at 0.1 vvm. The working volume was 1 L. The initial biomass concentration was 0.05 g L^{-1}. The culture medium was PM supplemented with glucose or DPPH. Depending on the experiment, the commercial yeast extract was replaced by SBYH. The repeated batch runs were performed in three stages. The biomass of the second and third stages was obtained by centrifugation of the cells suspended at the end of the previous stage.

2.4. Analytical methods

The biomass concentration was determined by UV-Vis spectrophotometry (Shimadzu UV-Vis spectrophotometer UV-1603), measuring the cell optical density at 600 nm.

The concentration values of the metabolites and the sugars of the substrate were obtained by high-performance liquid chromatography (HPLC) (Agilent Technologies 100 series), using a refractive index detector (RID) at 55 ºC. For the analysis of the sugars, a BP-800 Pb column (8%, 300 x 7.8 mm, Benson) was employed, while for the separation of the acids generated during the fermentation, a BP-800 H column (8%, 300 x 7.8 mm, Benson). Both columns worked at 80 ºC with a mobile phase of 5 Mm H_2SO_4 solution pumped at 0.5 mL min^{-1}.

2.5. Mathematical methods

For the estimation of the parameters of the kinetic model, the Aspen Custom Modeler v11 software (AspenTech, USA) was used, with an Euler method coupled to an adaptative non-linear least-square solver algorithm (NL2SOL), minimizing the difference between experimental values and kinetic model simulation data thanks to the least square method.

To determine the goodness of fit, the statistical parameters of equations (1-3) have been used. Therefore, estimates have been made in which Fisher's F-value (F) (Eq. 1) should be higher than its tabulated value at 95% confidence, the Root Mean Square Error (RMSE) (Eq. 2) should be as close to zero as possible and the Variation explained (VE) (Eq. 3) should be equal or near 100%.

$$F = \frac{\sum_{i=1}^{N}\left(\frac{y_{i,calc}}{K}\right)^2}{\sum_{i=1}^{N}\left(\frac{SSR}{N-K}\right)} \quad (1)$$

$$RMSE = \sqrt{\frac{SSR}{N-K}} \quad (2)$$

$$VE(\%) = 100\left(1 - \frac{\sum_{l=1}^{L} SSQ_l}{\sum_{l=1}^{L} SSQ_{mean_l}}\right) \quad (3)$$

In these equations, K was the number of parameters, N was the total number of experimental data, SSQ_l was the sum of the quadratic residues, SSQ_{meanl} was the squared sum of deviations between the experimental and the mean score with respect to the calculated values and SSR was the squared sum of residues:$\left(y_{i,exp} - y_{i,calc}\right)^2$, where $y_{i,exp}$ were the experimental values of the variable and $y_{i,calc}$ were the calculated values.

3. Results and discussion

Four experiments using repeated batch operation were carried out. One as a control (using commercial glucose and yeast extract) and, to determine the influence of each waste in the process the introduction of residues to replace carbon and nitrogen sources was carried out gradually. Therefore, first of all, two experiments were carried out, one in which the carbon source of the control experiment (glucose) was replaced by DPPH and the other, in which the carbon source used in the control experiment was maintained and the nitrogen source was modified, using SBYH. Finally, the experiment in which both nutrient sources were replaced by waste hydrolysates was carried out.

The values of the fermentation parameters (concentration, productivity, yield, selectivity) of all the experiments are shown in Table 1. In this table, we can observed that the production ratio between succinic acid and by-products remains constant during the first two stages (62 % selectivity), however, in the third stage an increase in the generation of by-products is observed (59 % selectivity). Moreover, the maximum yield is reached in the second stage (77% with respect to the glucose consumed). However, productivity increases at each cycle, from 0.99 g L-1 h-1 in the first cycle to a productivity of 3.1 g L-1 h-1 in the third cycle. Since the biomass increases throughout the cycles, the quantity of succinic acid produced does not suffer great variations, but its production rate does increase remarkably, the productivity in relation to the biomass concentration reaches its maximum in the second stage and the yield in relation to the biomass concentration is

maximum in the first stage, suggesting an activation of the biomass in the second stage (40-80% higher specific activity depending on the carbon and nitrogen source) and a progressive deactivation in the third cycle, more evident in the presence of the food waste hydrolysates.

Table 1. Concentrations, selectivity, yield and productivity on succinic acid production in repeated batch mode depending on the Carbon Source (CS) (glucose or DPPH) and the Nitrogen Source (NS) (yeast extract or SBYH).

	CS: GLUCOSE				**CS: DPPH**				
Cycle	1	2	3	Average	1	2	3	Average	
C_P ($g_P \cdot L^{-1}$)	23.8	25.4	24.8	24.7	27.7	28.1	31.1	28.8	**NS: YEAST EXTRACT**
S_P ($g_P \cdot g_{P+BP}^{-1}$)	0.62	0.62	0.59	0.61	0.78	0.77	0.74	0.76	
Y_P ($g_P \cdot g_{S.0}^{-1}$)	0.60	0.65	0.62	0.62	0.74	0.74	0.81	0.76	
$Y_{P/S.cons}$ ($g_P \cdot g_{S.cons}^{-1}$)	0.68	0.77	0.62	0.69	0.87	0.86	0.89	0.87	
$Y_{P/X}$ ($g_P \cdot g_X^{-1}$)	4.66	2.54	2.02	3.07	4.13	2.67	2.35	3.05	
P_P ($g_P \cdot L^{-1} \cdot h^{-1}$)	0.88	2.99	3.10	2.32	0.92	2.34	2.83	2.03	
$P_{P/X}$ ($g_P \cdot g_X^{-1} \cdot h^{-1}$)	0.17	0.30	0.25	0.24	0.14	0.22	0.21	0.19	
C_P ($g_P \cdot L^{-1}$)	29.1	32.2	33.1	31.5	30.2	34.7	35.8	33.6	**NS: SBYH**
S_P ($g_P \cdot g_{P+BP}^{-1}$)	0.78	0.79	0.74	0.77	0.80	0.81	0.78	0.80	
Y_P ($g_P \cdot g_{S.0}^{-1}$)	0.73	0.80	0.81	0.78	0.77	0.84	0.90	0.84	
$Y_{P/S.cons}$ ($g_P \cdot g_{S.cons}^{-1}$)	0.87	0.92	0.85	0.88	0.89	0.97	0.94	0.93	
$Y_{P/X}$ ($g_P \cdot g_X^{-1}$)	5.81	3.39	2.82	4.01	5.93	3.53	2.94	4.13	
P_P ($g_P \cdot L^{-1} \cdot h^{-1}$)	0.63	1.93	1.42	1.32	0.59	1.72	1.28	1.19	
$P_{P/X}$ ($g_P \cdot g_X^{-1} \cdot h^{-1}$)	0.13	0.20	0.12	0.15	0.12	0.17	0.11	0.13	

3.1. Succinic acid production in repeated batch mode with pure glucose and commercial YE.

As control test, the production of succinic acid from pure glucose and commercial yeast extract (YE) in a repeated batch operation was carried out, to assess the viability of using food wastes. Figure 1 shows the time course of the concentration of the substrate (S), succinic acid or product (P), by-products (BP) as the sum of acetic and formic acids, and biomass (X). In this Figure, BP (acetic and formic acids) were lumped because, as justified in previous works [30], both compounds follow the same growth trend, although acetic acid is generated in a greater proportion than formic acid.

It is observed that the products production is not growth-associated; in fact, in the first stage the biomass reaches a maximum concentration at 10 h: from that time it remains constant. In the following stages, the biomass does not stop growing, which may be due to the higher productivity of succinic acid in these stages, since they lead to shorter fermentation times, probably preventing the total consumption of nutrients. It must be pointed out the duration of each batch: the first one around 27 h, the second one around ten hours and the last one less than 10 h, requiring a total time of about 45 h. The increase in the glucose consumption is due to the cells being more adapted to the process as the number of stages in the repeated batch advances.

Figure 1

3.2. Succinic acid production in repeated batch mode with DPPH and commercial yeast extract

To verify the influence on the fermentation of the replacement of pure glucose by the potato hydrolysate, repeated batch experiments were carried out using this residue as a carbon source and commercial YE as the nitrogen source. The evolution of the biomass concentration and of the compounds involved in these reactions is shown in Figure 2. It can be observed that the introduction of this hydrolysate causes an increase in the total time for the completion of the three batch stages, requiring in this case around 55 h (22 % longer). From the values of the fermentation parameters shown in Table 1, it can be observed that yield, final succinic concentration and productivity increased at each stage, reaching a maximum of 89%, 31.1 g L^{-1} and 2.83 g L^{-1} h^{-1} respectively. Despite this, selectivity decreases at each stage of the process, going from 78 % in the first stage to 74 % in the third. In previous works [31] it was proven that the addition of adaptation stages to the carbon source favored the resistance of the microorganism to centrifugation and enabled the operation in resting state. Carrying out several stages of the process could resemble these mentioned adaptation stages and, therefore, be favorable to produce succinic acid. It is also observed that the yields and productivities in relation to the biomass concentration reach their maximum in the second stage.

Figure 2

3.3. Succinic acid production in repeated batch mode with pure glucose and SBYH

The following experiment was performed in order to determinate if yeast extract could be used as nitrogen source. Figure 3 shows the time course of glucose, biomass and products (main and by-products) when repeated batch is carried out using pure glucose and Spent Brewer's Yeast Hydrolysate (SBYH), as nitrogen source. The influence of replacing yeast extract with brewer's spent yeast hydrolysate is evident in the above figure, as the total time to carry out three batch steps doubles from about 45 h in Figure 1 to about 90 h in Figure 3. Regarding the values of the fermentation parameters listed in Table 1, as occurred with the reference experiment that used this same sugar (section 3.1), the maximum yield was reached in the second stage (92 %) and the same occurred with its productivity (1.93 g L^{-1} h^{-1}). The selectivity in the first two stages was very similar, around 78 %, while in the third stage it decreased to 74 %. Due to the increase in cell density throughout the stages, the maximum yield value in relation to the biomass concentration was achieved in the first stage. However, metabolic activity seems to have been maximum in the second stage, so the maximum productivity in relation to the cell concentration was achieved in the second. Despite using a nutrient source different from the two previous experiments (brewer's yeast hydrolysate instead of yeast extract), the biomass behavior was very similar in both cases, reaching a maximum after a few hours of fermentation. It remained constant until the beginning of the second, after which the cell concentration did not stop growing until the end of the fermentation.

Figure 3

3.4. Succinic acid production in repeated batch mode with DPPH and SBYH

Figure 4 shows the data when both hydrolysates were used as a carbon and nitrogen source simultaneously. It is observed that the time needed for three batch stages is around 100 hours, that is to say, the use of SBYH is the cause of the higher time needed for the process.

In this experiment, as shown in Table 1, the yield of the process is favored by carrying out successive fermentation stages. Again, as occurred with the experiments in which only glucose was replaced by DDPH (section 3.2), it seems that carrying out successive fermentation stages further favors the adaptation process of the microorganism to the carbon source, even after having performed previously, during the production of the inoculum, successive adaptations in shaken bottles. The maximum yield value was

reached in the last of the stages (90 % in relation to the substrate consumed). However, the production rate was higher in the second stage (1.72 g L^{-1} h^{-1}). The highest yield in relation to cell concentration was that corresponding to the first stage, while the maximum productivity in relation to biomass was obtained in the second stage.

Figure 4

The use of hydrolysates, both as a carbon and nitrogen source, seems to favor the yield of the process. In fact, when pure glucose and yeast extract were used, the overall yield of the process with respect to the consumed substrate was 69 %, while when it was simultaneously replaced by residues, this value increased to 93 %. However, the opposite occurred with productivity, in case of using commercial nutrients the average productivity was 2.32 g L^{-1} h^{-1}, while when DPPH and SBYH were used it decreased to 1.19 g L^{-1} h^{-1}. This loss in productivity was especially pronounced when replacing the nitrogen source, a 43 %, while a 12.5 % loss was obtained when replacing only the carbon source.

It should be noted that, in the two experiments in which commercial sugar was replaced by DPPH, the process was clearly favored by carrying out successive fermentation stages, despite the stress generated by centrifugation between stages, reaching maximum yield and productivity at the end of fermentation. However, in the case of using commercial YE, the highest yield was obtained in the second stage, but the production took place at a higher speed in the third stage. When pure glucose and SBYH were used, the opposite occurred, the highest yield was obtained at the end of the process, but at a lower speed than in the previous stage.

Table 2 shows the results of yield, productivity and final concentration of succinic acid achieved in this work in comparison with those obtained by other authors using the same biocatalyst. In this work, using repeated batch operations with food waste and free cells, higher yields (0.8 g g^{-1}) have been achieved than in repeated batch processes with immobilized cells reported in the literature, in which glucose (0.49 g g^{-1}) [26] or agave bagasse hydrolysate (0.39 g g^{-1}) [32] were used as carbon source and yeast extract as nitrogen source. The average and maximum productivities in repeated batch with yeast extract and glucose replaced by DPPH (2.03 g L^{-1} h^{-1} and 2.83 g L^{-1} h^{-1}) were similar and even higher when comparing the experiments with those of other authors in which only the pure sugars were substituted by hydrolysates, as in the studies carried out by Corona-González et al. [32] (1.10 g L^{-1} h^{-1} and 1.33 g L^{-1} h^{-1}). The highest productivity and yield

values in the literature were achieved with continuous operations in which the cells were in a stationary state of non-growth [26,33,34]. However, up to now, this type of operation with residues has not been carried out while there are hardly any studies in which the nitrogen source is replaced by residues. Among the few existing references, it is worth noting the work of Filippi et al. [22], who managed to carry out the production of succinic by simultaneously replacing the carbon and nitrogen source with winery residues in a fed-batch type operation, reaching yields and productivities of 0.64 g L^{-1} and 0.79 g L^{-1} h^{-1}, respectively.

303 Table 2. Summary of published studies on the production of succinic acid with *A.*
304 *succinogenes* depending on the type of operation and the carbon and nitrogen sources.

Type of operation	Cell state	Carbon source	Nitrogen source	Y_P ($g_P \cdot g_S^{-1}$)	P_P ($g_P \cdot L^{-1} \cdot h^{-1}$)	C_P ($g_P \cdot L^{-1}$)	Reference
Batch	Growing Free	Glucose	Yeast extract	0.63	0.13	20.8	[35]
Batch	Growing Free	Oil palm trunk	Yeast extract	0.47	0.18	10.6	[36]
Batch	Resting Free	Xylose	None	0.43	0.18	8.51	[31]
Batch	Growing Free	Grape Must	Yeast extract	0.67	0.45	43.2	[37]
Batch	Growing Free	Napier grass	Yeast extract	0.58	0.79	17.5	[38]
Repeated batch	Growing Immobilized	Glucose	Yeast extract	0.49*	~0.38*	~30*	[26]
Repeated batch	Growing Immobilized	Tequilana agave bagasse	Yeast extract	0.39*	1.10*	~6.72*	[32]
Continuous cell recycling reactor	Growing Free	Glucose	Yeast extract, Corn steep liquor	0.88	3.86	47.7	[33]
Continuous fluidized bed	Growing Immobilized	Glucose	Yeast extract	0.76	35.6	31	[26]
Continuous	Growing Immobilized	Glucose	Yeast extract, Corn steep liquor	0.68	3.4	29.4	[34]
Fed-batch	Growing Free	Citrus peel waste	Corn steep liquor, vitamins	0.73	0.45	22.4	[39]
Fed-batch	Growing Free	Olive pits	Yeast extract	0.27	0.50	33.7	[23]
Fed-batch	Growing Free	Grape pomace and stalks	Wine lees	0.64	0.79	37.2	[22]
Repeated batch	Free	Glucose	Yeast extract	0.62*	2.32*	24.7*	This study
Repeated batch	Free	DPPH	Yeast extract	0.76*	2.03*	28.8*	This study
Repeated batch	Free	Glucose	SBYH	0.78*	1.32*	31.5*	This study
Repeated batch	Free	DPPH	SBYH	0.84*	1.19*	33.6*	This study

305 * average values of all stages

306

3.5. Kinetic modelling of the bioprocess

To study the kinetics of the repeated batch fermentation from potato waste and spent brewer's yeast, a global and simple model has been applied, whose robustness was demonstrated in a previous work [30], through its application to batch type productions from glucose under different operating conditions and its application to a fed-batch fermentation.

It is a non-segregated unstructured model capable of accurately describing the evolution of glucose (S), succinic acid (P), by-products (SP) and biomass (X) with few parameters. Its reaction scheme is described in equations (4), (5) and (6). The reaction rates, r_i, are those corresponding to equations (7), (8) and (9), while the rates of substrate consumption and formation of products and biomass, R_j, are reflected in equations (10), (11), (12) and (13). C_{Xm} is the maximum biomass concentration, μ is the specific biomass growth rate, k_{P1} and k_{P2} are kinetic constants, and $Y_{S/X}$, $Y_{S/P1}$, $Y_{S/BP}$ and $Y_{S/P2}$ are macroscopic yields.

Reaction network:

$$Y_{S/X}S \xrightarrow{r_1} X \tag{4}$$

$$Y_{S/P1}S \xrightarrow{r_2} P + Y_{S/BP} \cdot BP \tag{5}$$

$$Y_{S/P2}S \xrightarrow{r_3} BP \tag{6}$$

Reaction rates:

$$r_1 = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_{Xm}}\right) \tag{7}$$

$$r_2 = k_{P1} \cdot C_S \cdot C_X \tag{8}$$

$$r_3 = k_{P2} \cdot C_S \cdot C_X \tag{9}$$

Production and consumption rates:

$$R_S = \frac{dC_S}{dt} = -Y_{S/X} \cdot r_1 - Y_{S/P1} \cdot r_2 - Y_{S/P2} \cdot r_3 \tag{10}$$

$$R_P = \frac{dC_P}{dt} = r_2 \tag{11}$$

$$R_X = \frac{dC_X}{dt} = r_1 \tag{12}$$

$$R_{BP} = \frac{dC_{BP}}{dt} = Y_{S/BP} \cdot r_2 + r_3 \tag{13}$$

We can appreciate in Table 3 and Figures 1, 2, 3 and 4, can be observed how the model fit very accurately to all relevant data. RMSE (0.81 – 2.07) and SSR (11.42 – 21.80) values are low while VE values (94.9 – 99.0%) are close to 100 % and Fisher's F values (1080 – 7648) are much higher than those needed to overcome the null hypothesis at 95 % (8.55 is the value of the F tabulated at that probability).

Table 3. Statistical parameter values calculated by fitting the kinetic model to experimental data on succinic acid production in repeated batch mode depending on the CS (glucose or DPPH) and the NS (yeast extract or SBYH).

	CS: GLUCOSE			**CS: DPPH**			
Cycle	1	2	3	1	2	3	
F	5238	3358	1967	2842	4959	1080	**NS: YEAST EXTRACT**
RMSE	1.07	1.14	1.30	0.86	0.98	2.07	
SSR	15.05	14.64	14.09	14.11	13.83	21.55	
VE (%)	98.7	97.7	98.6	99.0	98.4	94.9	
F	1695	7648	1518	1853	5313	2264	**NS: SBYH**
RMSE	1.37	0.81	1.97	1.41	1.08	1.86	
SSR	11.42	12.39	21.56	21.74	14.65	21.80	
VE (%)	97.3	99.3	96.9	97.3	98.7	97.2	

Table 4 compiles the kinetic parameters obtained and Figure 5 represents the parameters that undergo changes throughout the stages of repeated batch fermentation: the kinetic constants k_{P1} and k_{P2} and the parameters associated with biomass growth (C_{Xm} and μ). Regardless of the carbon or nitrogen source used, C_{Xm} increased throughout the three stages of the processes. However, in all cases, the growth rate of the biomass was much higher in the first stage in relation to the others, this fall in μ being especially pronounced in the experiments carried out with pure glucose as a carbon source and SBYH as a nitrogen source. The use of this hydrolysate as a nutrient led to very high biomass growth rates in the first stage, although to lower final biomass concentrations compared to processes in which the nitrogen source was yeast extract.

Figure 5

The constant k_{P1} followed similar trends to the productivities of the stages observed in Table 1. In the experiments in which yeast extract was used as the nitrogen source, the maximum productivity was reached in the third stage of the process, which is why a clearly increasing trend of k_{P1} is observed in both experiments. However, in the cases in which DDPH was used as nutrient, the maximum productivity was obtained in the second stage, observing a slight decrease in the third. This same evolution was reflected in the k_{P1} values throughout the three stages of these fermentations. Regarding k_{P2}, an increasing trend is observed in all cases because, in all of them, the selectivity was affected by the addition of stages in the process. The growth was especially accentuated in the cases in which yeast extract was used as the nitrogen source, due to the higher productivity of these fermentations.

Kinetic modelling is relevant not only to assess the effects of diverse nitrogen and carbon sources through lumped parameters that reflect the carbon use to diverse products but also as an starting point to perform technoeconomical and environmental studies. Here we appreciate activation and inhibition effects between cycles, which are different depending on the carbon and nitrogen sources employed. Li et al. recently observed the substrate (glycerol) and product (mainly, acetic acid) inhibitory effects by modelling their results using a Monod model with substrate and product inhibition using the yeast *Yarrowia lipolytica* and numerous data obtained in shaken flasks [40]. As in this work, we have observed that most carbon is derived towards the production of succinic acid than to biomass and by-products according to the values of the relevant kinetic parameters, which also reflect the effect of the use of hydrolysates resulting in a higher selectivity to succinic acid with food waste hydrolysates. In fact, when using the mixture glucose/yeast extract the k_{p1}/k_{p2} ratio is 3.16 while this same ratio for the DPPH/SBYH mixture is 8.1. It is interesting to observe that is the substitution of the nitrogen source (SBYH instead of yeast extract) the factor that most notably enhances succinic acid selectivity (k_{p1}/k_{p2}=6.8), while changing the carbon source from glucose to the potato hydrolysate DPPH only increases this ratio marginally (3.6 instead of 3.16). The combination of waste hydrolysates with an adequate pH control strategy can also result in a notable increase of the yield to succinic acid, as appreciated by Li et al. [41]. In addition, we should remember that succinic acid production is also a CO_2 sink due to the fixation of one additional carbon atom per acid molecule, so, all in all, the use of waste hydrolysates and this reality is the at the back of sustainable process design, as clearly pointed out by Bello et al. [42].

Table 3. Kinetic parameter values calculated by fitting the kinetic model to experimental data on succinic acid production in repeated batch mode depending on the carbon source (CS) (glucose or DPPH) and the nitrogen source (NS) (yeast extract or SBYH).

	CS: GLUCOSE			**CS: DPPH**			
Cycle	1	2	3	1	2	3	
C_{Xm} (g L^{-1})	5.12 ± 0.06	10.1 ± 0.43	12.2 ± 0.77	6.71 ± 0.25	1.05 ± 0.05	1.32 ± 0.06	**NS: YEAST EXTRACT**
kp_1 (L g^{-1} h^{-1})	(1.17 ± 0.09)· 10^{-2}	(1.85 ± 0.05) · 10^{-2}	(2.02 ± 0.16)· 10^{-2}	(1.01 ± 0.07)· 10^{-2}	(1.37 ± 0.09) · 10^{-2}	(1.31 ± 0.0) · 10^{-2}	
kp_2 (L g^{-1} h^{-1})	(3.70 ± 0.28) · 10^{-3}	(4.35 ± 0.28)· 10^{-3}	(7.33 ± 0.46)· 10^{-3}	(2.80 ± 0.18)· 10^{-3}	(3.80 ± 0.21) · 10^{-3}	(4.28 ± 0.28) · 10^{-3}	
μ (h^{-1})	(8.45 ± 0.19) · 10^{-1}	(3.45 ± 0.16) · 10^{-1}	(3.45 ± 0.16)· 10^{-1}	(7.82 ± 0.28)· 10^{-1}	(2.28 ± 0.12) · 10^{-1}	(2.07 ± 0.09) · 10^{-1}	
$Y_{S/P1}$ (g g^{-1})	(6.62 ± 0.51)· 10^{-1}	(6.62 ± 0.51)· 10^{-1}	(6.62 ± 0.51)· 10^{-1}	(9.68 ± 0.42) · 10^{-1}	(9.68 ± 0.42) · 10^{-1}	(9.68 ± 0.42) · 10^{-1}	
$Y_{S/P2}$ (g g^{-1})	2.20 ± 0.18	2.20 ± 0.18	2.20 ± 0.18	(2.43 ± 0.20) · 10^{-1}	(2.43 ± 0.20) · 10^{-1}	(2.43 ± 0.20) · 10^{-1}	
$Y_{S/BP}$ (g g^{-1})	(3.29 ± 0.21) · 10^{-1}	(3.29 ± 0.21) · 10^{-1}	(3.29 ± 0.21) · 10^{-1}	(8.41 ± 0.62) · 10^{-3}	(8.41 ± 0.62) · 10^{-3}	(8.41 ± 0.62) · 10^{-3}	
$Y_{S/X}$ (g g^{-1})	(2.89 ± 0.19) · 10^{-1}	(2.89 ± 0.19) · 10^{-1}	(2.89 ± 0.19) · 10^{-1}	(4.17 ± 0.27) 10^{-1}	(4.17 ± 0.27) 10^{-1}	(4.17 ± 0.27) 10^{-1}	
C_{Xm} (g L^{-1})	5.00 ± 0.23	9.48 ± 0.52	1.18 ± 0.05	5.10 ± 0.19	9.83 ± 0.46	1.22 ± 0.07	**NS: SBYH**
kp_1 (L g^{-1} h^{-1})	(6.62 ± 0.36)· 10^{-3}	(1.24 ± 0.08) · 10^{-2}	(8.50 ± 0.63) · 10^{-3}	(6.00 ± 0.32) · 10^{-3}	(1.02 ± 0.08) · 10^{-2}	(7.45 ± 0.63) · 10^{-3}	
kp_2 (L g^{-1} h^{-1})	(9.73 ± 0.82) · 10^{-4}	(1.71 ± 0.13) · 10^{-3}	(1.80 ± 0.15) · 10^{-3}	(7.41 ± 0.62) · 10^{-4}	(9.84 ± 0.08) · 10^{-4}	(1.06 ± 0.08) · 10^{-3}	
μ (h^{-1})	1.21 ± 0.06	(1.84 ± 0.08) · 10^{-1}	(1.17 ± 0.06) · 10^{-1}	1.04 ± 0.06	(1.47 ± 0.08) · 10^{-1}	(9.32 ± 0.04) · 10^{-2}	
$Y_{S/P1}$ (g g^{-1})	(8.62 ± 0.56) · 10^{-1}	(8.62 ± 0.56) · 10^{-1}	(8.62 ± 0.56) · 10^{-1}	(7.86 ± 0.29) · 10^{-1}	(7.86 ± 0.29) · 10^{-1}	(7.86 ± 0.29) · 10^{-1}	
$Y_{S/P2}$ (g g^{-1})	1.21 ± 0.09	1.21 ± 0.09	1.21 ± 0.09	1.87 ± 0.08	1.87 ± 0.08	1.87 ± 0.08	
$Y_{S/BP}$ (g g^{-1})	(1.36 ± 0.12) · 10^{-1}	(1.36 ± 0.12) · 10^{-1}	(1.36 ± 0.12) · 10^{-1}	(1.32 ± 0.07) · 10^{-1}	(1.32 ± 0.07) · 10^{-1}	(1.32 ± 0.07) · 10^{-1}	
$Y_{S/X}$ (g g^{-1})	(2.61 ± 0.17) · 10^{-1}	(2.61 ± 0.17) · 10^{-1}	(2.61 ± 0.17) · 10^{-1}	(2.01 ± 0.13) · 10^{-1}	(2.01 ± 0.13) · 10^{-1}	(2.01 ± 0.13) · 10^{-1}	

4. Conclusions

It is possible to carry out a successful bioproduction of succinic acid through the action of *A. succinogenes* from only food residues as carbon and nitrogen source, specifically, residues of discarded potato pieces and spent brewer's yeast respectively. This implies taking one more step towards a circular economy in which wastes are reused as secondary raw material in biorefinery processes, drastically reducing the costs associated to fresh raw materials. The study shows that the use of these residues in repeated batch leads to 35% higher yield values than its equivalent process with commercial glucose and yeast extract. However, these residues seem to have the opposite effect on reaction times, prolonging them considerably, especially in those cases where spent brewer's yeast was used as the nitrogen source. A unstructured non-segregated kinetic model, with few parameters and high goodness of fit, was successfully applied to all stages of the repeated batch operation, regardless of the type of carbon and nitrogen source used, helping in a future techno-economic analysis or process scale-up.

Declaration of Competing Interest

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

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Figure captions

Figure 1. Evolution of substrate (S), succinic acid (P), by-products (BP, acetic and formic acids) and biomass (X) employing pure glucose as carbon source and commercial yeast extract as nitrogen source. Data points: (●) S, (■) P, (▲) BP, (▼) X. Lines: model prediction.

Figure 2. Evolution of substrate (S), succinic acid (P), by-products (BP, acetic and formic acids) and biomass (X) employing DPPH as carbon source and commercial yeast extract as nitrogen source. Data points: (●) S, (■) P, (▲) BP, (▼) X. Lines: model prediction.

Figure 3. Evolution of substrate (S), succinic acid (P), by-products (BP, acetic and formic acids) and biomass (X) employing pure glucose as carbon source and SBYH as nitrogen source. Data points: (●) S, (■) P, (▲) BP, (▼) X. Lines: model prediction.

Figure 4. Evolution of substrate (S), succinic acid (P), by-products (BP, acetic and formic acids) and biomass (X) employing DPPH as carbon source and SBYH as nitrogen source. *Data points:* (●) S, (■) P, (▲) BP, (▼) X. Lines: model prediction.

Figure 5. Kinetic parameters that are modified during the three stages of repeated batch production. Key: (red bars) glucose as CS and yeast extract as NS, (green bars) DPPH as CS and yeast extract as NS, (blue bars) glucose as CS and SBYH as NS, (purple bars) DDPH as CS and SBYH as NS.

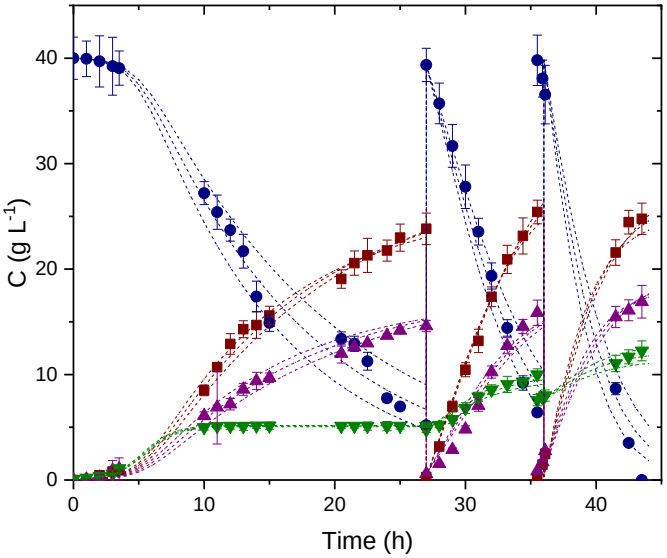
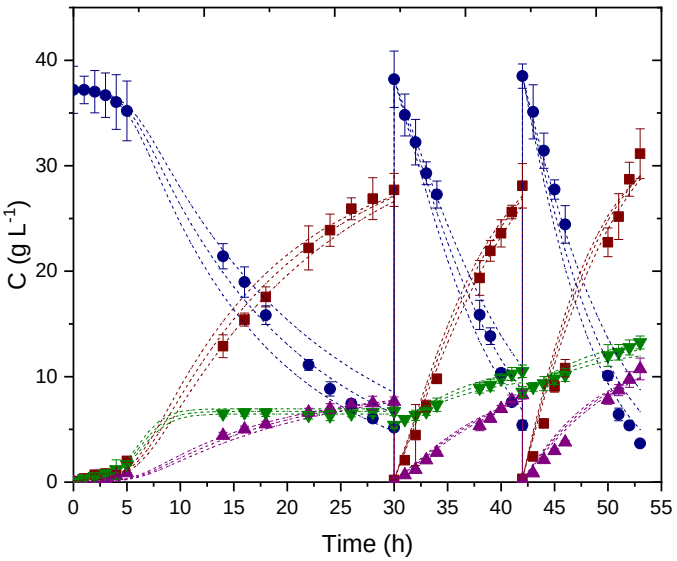


Figure 1.



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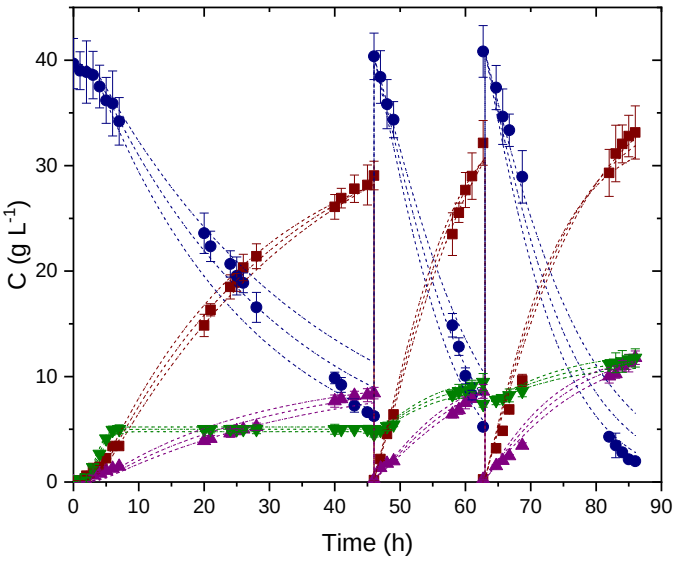

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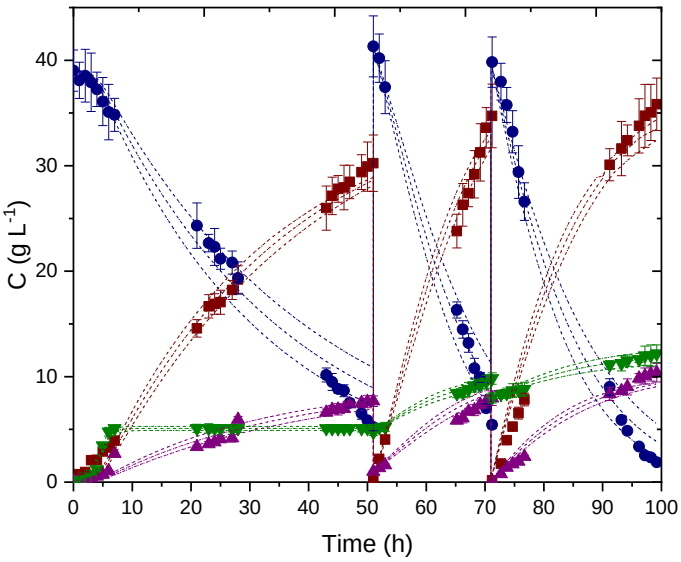

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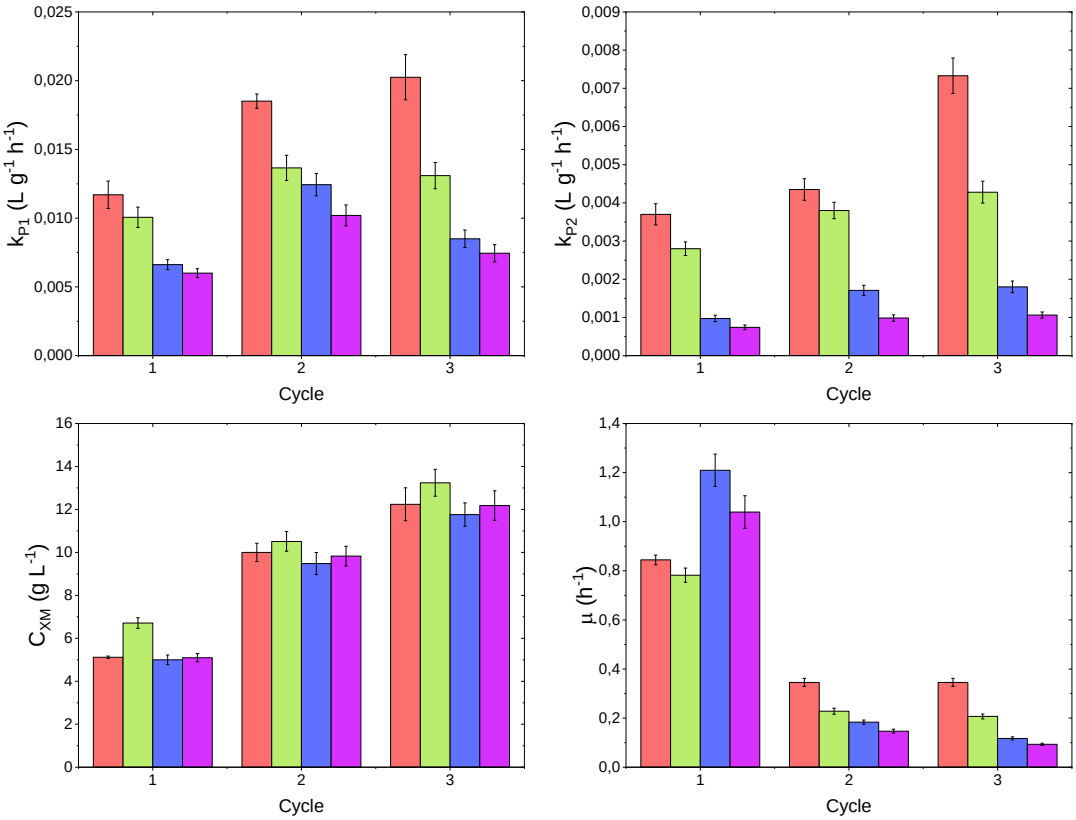
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0,007
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0,004
0,003
0,002
0,001
0,000
k_{P2} (L g^{-1} h^{-1})
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Cycle
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2
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3
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3
Cycle

Figure 5.

Declaration of Competing Interest

The authors declare that they have no knowledge of competing financial interests or personal relationships that could have influenced or appeared to influence the work here reported.