CLOSTRIDIUM FERMENTATION PLATFORMS FOR THE PRODUCTION OF SECOND-GENERATION BIOALCOHOLS FROM RICE STRAW

Miguel Capilla Lloris

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Supervisors:

Dr. Carmen Gabaldón García
Dr. Pau San Valero Tornero

VNIVERSITAT DE VALÈNCIA

Escola Tècnica Superior d'Enginyeria
Departament d'Enginyeria Química

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Memoria que, para optar al Título de Doctor por la Universitat de València, presenta **MIGUEL CAPILLA LLORIS**

Directoras de tesis,
Dra. **CARMEN GABALDÓN GARCÍA**
Dra. **PAU SAN VALERO TORNERO**

València, julio de 2023

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

Dra. PAU SAN VALERO TORNERO, Profesora Titular del Departamento de Ingeniería Química de la Universitat de València

CERTIFICAN: Que **Miguel Capilla Lloris**, graduado en Biotecnología y con título de Máster en Microbiología Aplicada, ha realizado bajo su dirección el trabajo que bajo el título de: **"*Clostridium* fermentation platforms for the production of second-generation bioalcohols from rice straw"** presenta en esta Memoria y que constituye su Tesis para optar al Título de Doctor por la Universitat de València en el Programa de Doctorado en Ingeniería Química, Ambiental y de Procesos.

Y para que conste a los efectos oportunos firman el presente certificado en Valencia a julio de 2023.

Fdo.: Dra. Carmen Gabaldón García Fdo.: Dra. Pau San Valero Tornero

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RESUMEN

El cambio climático constituye una gran amenaza que está obligando a los gobiernos a fomentar políticas activas para disminuir las emisiones de gases de efecto invernadero. La Unión Europea ha promulgado el “Pacto Verde Europeo” que pretende alcanzar cero emisiones netas en 2050. En este contexto, el desarrollo de biorefinerías para producir bioalcoholes por vía fermentativa utilizando como materia prima residuos lignocelulósicos contribuiría a alcanzar el objetivo de la neutralidad climática. Entre las rutas que destacan se encuentran la fermentación acetona-butanol-etanol (ABE) o la fermentación hexanol-butanol-etanol (HBE) ambas llevadas a cabo por bacterias del género *Clostridium*.

El objetivo general de la presente tesis doctoral es la producción de biobutanol y biohexanol mediante fermentación utilizando bacterias del género *Clostridium* con el propósito de investigar nuevas vías de aprovechamiento de los residuos lignocelulósicos. En concreto, se ha seleccionado la paja de arroz debido a su disponibilidad y abundancia en el Parque Natural de l’Albufera (Valencia, España), uno de los mayores humedales de la cuenca mediterránea donde se cultiva un 18% de la producción española de arroz.

La mayor parte de esta tesis doctoral se centró en la producción de butanol via fermentación ABE a partir de paja de arroz utilizando *Clostridium acetobutylicum*. En el primer trabajo presentado en esta tesis doctoral se evaluó el efecto combinado de la concentración inicial de glucosa y la estrategia de regulación de pH en fermentaciones en discontinuo. En esta etapa se compararon dos estrategias de regulación de pH consistentes en la formulación del medio de cultivo con carbonato de calcio como agente tamponante y una estrategia de control digital consistente en impedir que el pH disminuyera por debajo de un valor de consigna. Con este estudio se demostró la importancia de regular el pH *ad hoc* en función de la concentración inicial de azúcar. En concreto, la utilización de la estrategia de control de pH mínimo para concentraciones iniciales de glucosa ≥66 g L^{-1} permitió evitar el fenómeno de choque ácido obteniéndose una concentración de butanol de ~11 g L^{-1}.

En el siguiente estudio se amplió la evaluación de las estrategias de regulación de pH desarrolladas anteriormente a xilosa y mezclas de glucosa y xilosa. Los resultados demostraron que *C. acetobutylicum* se comporta de forma diferente cuando se utiliza xilosa pura como fuente de carbono. En este caso, la producción de biobutanol se vio favorecida al no limitarse el pH mínimo de la fermentación dando lugar a

concentraciones de butanol de ~8 g L^{-1}, límite de inhibición al emplear este monosacárido. Para el caso de mezclas de glucosa y xilosa en proporciones similares a las obtenidas en los hidrolizados de paja de arroz se demostró que la estrategia más eficaz en términos de producción de butanol y consumo de xilosa es la formulación de medio con acetato amónico y un control de pH mínimo a 4.8. En la siguiente etapa, se desarrolló una prueba de concepto de un reactor en continuo de tanque agitado con un sistema de retención de biomasa utilizando anillos plásticos con el objetivo de incrementar la productividad de butanol. Para la mezcla de glucosa y xilosa y operando el reactor a una velocidad de dilución de 0.333 h^{-1} se cuadruplicaron las productividades de butanol y disolventes ABE (butanol: 2.4 g L^{-1} h^{-1} y disolventes ABE: 3.7 g L^{-1} h^{-1}) al compararlas con los mejores resultados de los experimentos en condiciones similares en discontinuo (butanol: 0.6 g L^{-1} h^{-1} y disolventes ABE: 0.9 g L^{-1} h^{-1}).

En el siguiente trabajo de esta tesis doctoral se validó el modelo de fermentación en discontinuo usando hidrolizado de paja de arroz. Este hidrolizado se obtuvo a partir de paja de arroz pretratada alcalinamente. En estos ensayos se logró una producción de butanol (disolventes ABE) de 6.5 (9.5) g L^{-1}, consiguiendo resultados similares a los a los obtenidos con sustrato modelo. A continuación, se evaluó el empleo de una estrategia de co-cultivo de *C. acetobutylicum* y *Saccharomyces cerevisiae*, obteniéndose un incremento en la producción de disolventes ABE hasta 13.1 g L^{-1} asociado mayoritariamente a la producción de etanol. Además, se obtuvo un aprovechamiento de xilosa del 94%, el mayor observado en esta tesis doctoral.

Finalmente, se realizó una estancia internacional de tres meses en la *Università degli Studi di Napoli Federico II* (Italia) donde se estudió la producción de bioalcoholes en continuo vía fermentación HBE a partir de corrientes gaseosas de monóxido de carbono, principal componente del gas de síntesis. En estos experimentos se desarrolló un reactor en continuo de tanque agitado en el que se evaluó la influencia del pH empleando *Clostridium carboxidivorans*. En estos experimentos se obtuvo un incremento de la productividad de hexanol de 1.8 al utilizar un valor de pH 5.6 respecto a un valor de pH 5.4. Además, la utilización de un valor de pH 5.6 resultó en productividades estables de hexanol entre 100 a 140 mg L^{-1} dia^{-1} en el rango de 0.034 a 0.083 h^{-1}. Estas productividades se encuentran entre los valores más elevados de la bibliografía.

RESUM

El canvi climàtic constitueix una gran amenaça que està obligant als governs a fomentar polítiques actives per a disminuir les emissions de gasos d'efecte d'hivernacle. La Unió Europea ha promulgat el "Pacte Verd Europeu" que pretén aconseguir zero emissions netes en 2050. En aquest context, el desenvolupament de biorefineries per a produir bioalcohols per via fermentativa utilitzant com a matèria primera residus lignocel·lulòsics contribuiria a aconseguir l'objectiu de la neutralitat climàtica. Entre les rutes que destaquen es troben la fermentació acetona-butanol-etanol (ABE) o la fermentació hexanol-butanol-etanol (HBE) totes dues dutes a terme per bacteris del gènere *Clostridium*.

L'objectiu general de la present tesi doctoral es la producció de biobutanol i biohexanol mitjançant fermentació utilitzant bacteris del gènere *Clostridium* amb el propòsit d'investigar noves vies d'aprofitament dels residus lignocel·lulòsics. En concret, s'ha seleccionat la palla d'arròs degut a la seua disponibilitat i abundància al Parc Natural de l'Albufera (València, Espanya), un dels majors aiguamolls de la conca mediterrània on es cultiva un 18% de la producció espanyola d'arròs.

La major part d'aquesta tesi doctoral es va centrar en la producció de butanol via fermentació ABE a partir de palla d'arròs utilitzant *Clostridium acetobutylicum*. En el primer treball presentat en aquesta tesi doctoral es va avaluar l'efecte combinat de la concentració inicial de glucosa i l'estratègia de regulació de pH en fermentacions en discontinu. En aquesta etapa es van comparar dues estratègies de regulació de pH consistents en la formulació del medi de cultiu amb carbonat de calci com a agent tamponant i una estratègia de control digital consistent a impedir que el pH disminuïra per davall d'un valor de consigna. Amb aquest estudi es va demostrar la importància de regular el pH *ad hoc* en funció de la concentració inicial de sucre. En concret, la utilització de l'estratègia de control de pH mínim per a concentracions inicials de glucosa ≥66 g L^{-1} va permetre evitar el fenomen de xoc àcid obtenint-se una concentració de butanol de ~11 g L^{-1}.

En el següent estudi es va ampliar l'avaluació de les estratègies de regulació de pH desenvolupades anteriorment a xilosa i mescles de glucosa i xilosa. Els resultats van demostrar que *C. acetobutylicum* es comporta de manera diferent quan s'utilitza xilosa pura com a font de carboni. En aquest cas, la producció de biobutanol es va veure afavorida al no limitar-se el pH mínim de la fermentació donant lloc a concentracions de butanol de ~8 g L^{-1}, límit d'inhibició en emprar aquest monosacàrid. Per al cas de

mescles de glucosa i xilosa en proporcions similars a les obtingudes en els hidrolitzats de palla d'arròs es va demostrar que l'estratègia més eficaç en termes de producció de butanol i consum de xilosa és la formulació de medi amb acetat amònic i un control de pH mínim a 4.8. En la següent etapa, es va desenvolupar una prova de concepte d'un reactor en continu de tanc agitat amb un sistema de retenció de biomassa utilitzant anells plàstics amb l'objectiu de incrementar la productivitat de butanol. Per a la mescla de glucosa i xilosa i operant el reactor a una velocitat de dilució de 0.333 h^{-1} es van quadruplicar les productivitats de butanol i dissolvents ABE (butanol: 2.4 g L^{-1} h^{-1} i dissolvents ABE: 3.7 g L^{-1} h^{-1}) en comparar-les amb els millors resultats dels experiments en condicions similars en discontinu (butanol: 0.6 g L^{-1} h^{-1} i dissolvents ABE: 0.9 g L^{-1} h^{-1}).

En el següent treball d'aquesta tesi doctoral, es va validar el model de fermentació en discontinu usant hidrolitzat de palla d'arròs. Aquest hidrolitzat es va obtindre a partir de palla d'arròs pretratada alcalinament. En aquests assajos es va assolir una producció de butanol (dissolvents ABE) de 6.5 (9.5) g L^{-1}, aconseguint resultats similars als obtinguts amb substrat model. A continuació, es va avaluar l'ús d'una estratègia de co-cultiu de *C. acetobutylicum* i *Saccharomyces cerevisiae*, obtenint-se un increment en la producció de dissolvents ABE fins a 13.1 g L^{-1} associat majoritàriament a la producció d'etanol. A més, es va obtindre un aprofitament de xilosa del 94%, el major observat en aquesta tesi doctoral.

Finalment, es va realitzar una estada internacional de tres mesos en la *Università degli Studi di Napoli Federico II* (Itàlia) on es va estudiar la producció de bioalcohols en continu via fermentació HBE a partir de corrents gasosos de monòxid de carboni, principal component del gas de síntesi. En aquests experiments es va desenvolupar un reactor en continu de tanc agitat en el qual es va avaluar la influència del pH emprant *Clostridium carboxidivorans*. En aquests experiments es va obtindre un increment de la productivitat d'hexanol d'1.8 en utilitzar un valor de pH 5.6 respecte a un valor de pH 5.4. A més, la utilització d'un valor de pH 5.6 va resultar en productivitats estables de hexanol entre 100 a 140 mg L^{-1} dia^{-1} en el rang de 0.034 a 0.083 h^{-1}. Aquestes productivitats es troben entre els valors més elevats de la bibliografia.

SUMMARY

Climate change is a major threat that is forcing governments to promote active policies to reduce greenhouse gas emissions. The European Union has enacted the "European Green Deal" which aims to achieve net zero emissions by 2050. In this context, the development of biorefineries to produce bioalcohols by fermentation using lignocellulosic waste as feedstock would contribute to achieve the goal of climate neutrality. Among the routes that stand out are acetone-butanol-ethanol (ABE) or hexanol-butanol-ethanol (HBE) fermentation, both carried out by bacteria of the genus *Clostridium*.

The general objective of this doctoral thesis is the production of biobutanol and biohexanol by fermentation using bacteria of the genus *Clostridium* with the aim of investigating new ways of exploiting the lignocellulosic waste. Specifically, rice straw has been selected due to its availability and abundance in l'Albufera Natural Park (Valencia, Spain), one of the largest wetlands in the Mediterranean basin where 18% of Spanish rice production is harvested.

The main part of this doctoral thesis focused on the production of butanol via ABE fermentation from rice straw using *Clostridium acetobutylicum*. The first work presented in this doctoral thesis evaluated the combined effect of the initial glucose concentration and the pH regulation strategy in batch fermentations. In this stage, two pH regulation strategies were compared, consisting of the formulation of the culture media with calcium carbonate as a buffering agent and a digital control strategy consisting of preventing the pH from decreasing below a set-point value. This study demonstrated the importance of *ad hoc* pH regulation depending on the initial sugar concentration. In particular, the use of the minimum pH control strategy for initial glucose concentrations ≥66 g L^{-1} allowed to avoid the acid crash phenomenon obtaining a butanol concentration of ~11 g L^{-1}.

In the following study, the evaluation of the previously developed pH regulation strategies was extended to xylose and glucose and xylose mixtures. The results showed that *C. acetobutylicum* behaves differently when pure xylose is used as a carbon source. In this case, biobutanol production was enhanced by not limiting the minimum pH of the fermentation, leading to butanol concentrations of ~8 g L^{-1}, which is the inhibition limit at the use of this monosaccharide. In the case of glucose and xylose mixtures in similar proportions to those obtained in rice hydrolysates, it was demonstrated that the most efficient strategy in terms of butanol production and xylose consumption is the media formulation with ammonium acetate and a minimum pH control at 4.8. In the next stage,

a proof of concept of a continuous stirred tank reactor with a biomass retention system using plastic rings was developed in order to increase butanol productivity. For the mixture of glucose and xylose and operating the reactor at a dilution rate of 0.333 h^{-1} the productivities of butanol and ABE solvents (butanol: 2.4 g L^{-1} h^{-1} and ABE solvents: 3.7 g L^{-1} h^{-1}) were quadrupled when compared to the best results of experiments under similar batch conditions (butanol: 0.6 g L^{-1} h^{-1} and ABE solvents: 0.9 g L^{-1} h^{-1}).

In the following work of this doctoral thesis, the batch fermentation model was validated using rice straw hydrolysate. This hydrolysate was obtained from alkaline pretreated rice straw. In these assays, a butanol production (ABE solvents) of 6.5 (9.5) g L^{-1} was reached, achieving similar results to those obtained with the model substrate. Next, the use of a co-culture strategy of *C. acetobutylicum* and *Saccharomyces cerevisiae* was evaluated, obtaining an increase in the ABE solvent production up to 13.1 g L^{-1} associated mainly with the ethanol production. In addition, a xylose exploitation of 94% was obtained, the highest observed in this doctoral thesis.

Finally, a three-month international stay was carried out at the *Università degli Studi di Napoli Federico II* (Italy) where the continuous production of bioalcohols via HBE fermentation from carbon monoxide gas streams, the main component of synthesis gas, was studied. In these experiments, a continuous stirred tank reactor was developed in which the influence of pH was evaluated using *Clostridium carboxidivorans*. In these experiments, an increase in hexanol productivity of 1.8 was obtained when using a pH value of 5.6 compared to a pH value of 5.4. Furthermore, the use of a pH value of 5.6 resulted in stable hexanol productivities between 100 to 140 mg L^{-1} day^{-1} in the range of 0.034 to 0.083 h^{-1}. These productivities are among the highest values in the literature.

INDEX

1. THESIS OVERVIEW

The increment of greenhouse gasses emissions from the anthropogenic activities of the last century due to the use of fossil fuels has raised the interest of new sources of biobased chemicals. The development of second generation bioalcohols from non-edible feedstocks aims to mitigate the effects of climate change. This PhD thesis deals with the production of biobutanol and biohexanol by solventogenic fermentation from *Clostridium* spp. The PhD book consists of 11 chapters and is prepared as a compendium of three scientific publications constituting Chapters 6 to 8 plus Chapter 9 compiled from the results of a three-month research stay. Chapters 6 to 8 are an adaptation of the original manuscripts already published. The results from a three-month research stay were obtained at the *Università degli Studi di Napoli Federico II* (Italy) under the supervision of Dr. Francesca Raganati. After outlining the structure of the PhD thesis herein, Chapter 2 depicts a general introduction of the topic on the development of second-generation bioalcohols while Chapter 3 emphasizes on the production of biosolvents by solventogenic *Clostridium* spp. Chapter 4 introduces the objectives of this PhD thesis and Chapter 5 details a summary of the materials and methods employed in this work. The conclusions obtained during the span of the PhD thesis are detailed together with the future perspectives in Chapter 10. Chapter 11 comprises an extended summary in Spanish compulsory according to the regulations of the University of Valencia. The appendix section is composed by the glossary of abbreviations and terms (Appendix A.1) and the published papers with their supplementary materials (Appendix A.2).

The complete references of the scientific publications are the following:

- Paper I (Chapter 6): Capilla, M., San-Valero, P., Izquierdo, M., Penya-roja, J.M., Gabaldón, C., 2021. The combined effect on initial glucose concentration and pH control strategies for acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* DSM 792. Biochem. Eng. J. 167, 107910. https://doi.org/10.1016/j.bej.2020.107910

- Paper II (Chapter 7): Capilla, M., Silvestre, C., Valles, A., San-Valero, P., Álvarez-Hornos, F.J., Gabaldón, C., 2022. The influence of sugar composition and pH regulation in batch and continuous acetone-butanol-ethanol fermentation. Fermentation. 8, 226. https://doi.org/10.3390/fermentation8050226

- Paper III (Chapter 8): Capilla, M., Valles, A., San-Valero, P., Álvarez-Hornos, F.J., Gabaldón, C., 2022. Solvent production from rice straw by a co-culture of *Clostridium acetobutylicum* and *Saccharomyces*

cerevisiae: effect of pH control. Biomass Convers. https://doi.org/10.1007/s13399-022-02750-4

Elsevier Ltd., MDPI and Springer have given the rights to include these papers in this PhD thesis.

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2. SECOND-GENERATION BIOALCOHOLS

2.1. Second-generation biorefineries as part of the solution to mitigate climate change

The concentrations of greenhouse gases (GHGs) have risen since 1750 due to anthropogenic activities. From 1750 over the 2019, the concentrations of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) have increased in 47% (to 410 parts per million, ppm), 156% (to 1866 parts per billion, ppb) and 23% (to 332 ppb), respectively. The Intergovernmental Panel on Climate Change's (IPCC) Sixth Assessment Report has corroborated the unequivocal influence of the human being in the warming of the atmosphere, ocean, and land. Considering the global surface temperature of the decade of 2011-2020, it has increased an average of 1.09 ºC over the mid-19th century. It is expected that the temperature of the planet will rise about 1.5-2.0 ºC during the 21st century unless huge reductions on GHG emission happens in the following years (IPCC, 2021).

The magnitude and scale of the climate change will depend on the adoption of early mitigation and adaptation actions, increasing the complexity of the situation if these acts are postponed to mid- or long-term (IPCC, 2022, 2021). There are achievable and fruitful adaptation approaches that can reduce some human- and nature-related risks to the climate change which are not directly related with the GHG reductions (IPCC, 2022). However, a fast and intense reduction on the emission of these gases is also necessary to restrain the climate change. Several scenarios have been estimated depending on the predicted cuts of GHGs. Among them, the most favourable approximation would lead to an average increase of 1.0-1.8 ºC, while the worst scenario would result in a 3.3-5.7 ºC long-term increment (2081-2100) (IPCC, 2021). Recent estimations show that achieving global net zero CO_2 emissions in the 2050s would limit the temperature increase in 1.5 ºC (IPCC, 2022). Hence to restrain the anthropogenic climate change is necessary to achieve, at least, net zero CO_2 emissions. Moreover, this action alongside with the reduction of other GHGs, such as, CH_4 or aerosol emissions would improve the quality of the air (IPCC, 2021).

Following with the reduction of GHGs, a practical example can be introduced due to the emerging of the SARS-CoV-2 (COVID-19). On the 11st of March of 2021 the World Health Organization declared the global pandemic of COVID-19 leading to a cease of global activities during part of the year 2020. The quarantine imposed by the countries to stop the spread of COVID-19 led to a reduction of 7% in CO_2 emissions compared to 2019, mainly from cuttings in the transport sector. Nevertheless, this reduction on the GHG emissions was not permanent after the lifting of the restriction policies. Permanent

reductions on GHG emissions would require a change of paradigm on the global economic framework (Le Quéré et al., 2021, 2020).

In this context, there is a need of active and sharp government policies to achieve carbon neutrality and to reduce the effect of the climate change. The European Union is committed on this fight, setting a closer objective in 2030 to reduce the GHG emissions by a 55% compared to the 1990 levels. On the 11th of December 2019, the European Commission launched the "The European Green Deal" which is set to regulate the transformation of the European Union into a decoupled society from resource use along with a net zero GHG emission region by 2050 (European Union: European Commission, 2019). On 2021, the United Nations celebrated its Climate Change Conference (COP26) in Glasgow, where the following outcomes were achieved: 1) the recognition of the climate emergency; 2) the limitation of the average temperature's increment to 1.5 ºC; 3) the acceleration of the mitigation actions to climate change; 4) the finance of the developing countries to help them on the adaptation to climate change impacts; 5) the completion of the Paris Agreement rulebook and 6) the cease of the use of the fossil fuels (UNFCCC, 2022). Moreover, the European Commission approved the Regulation 2021/1119 on the 30th June for establishing the framework to achieve climate neutrality ("European Climate Directive") (European Commission, 2021). Other countries are also implementing policies on the mitigation of climate change. The United States of America (USA) is working to be net zero GHG emissions by 2050 (United States Department of State, 2021) and China's policies aim to reach the peak of CO_2 production before 2030 and become carbon neutral by 2060 (The People's Republic of China, 2021).

Among the strategies to combat climate change, the shifting to a circular economy would help to reduce the utilization of non-renewable carbon sources. The circular economy is a model of manufacturing and utilization that aims to share, reuse, repair and recycle goods as many times as possible, therefore extending the life cycle of consumables. It involves cutting down the residues to a minimum and whenever possible, maintaining those residues into a closed-economy instead of a linear use model (Figure 2.1). The circular economy encloses several options to reduce the extensive use of non-renewable feedstocks. In this regard, biorefineries were defined as *"The sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)"* by the International Energy Agency (IEA) (IEA-Bioenergy, 2020). This alternative included in the circular economy can treat a wide range of feedstocks originated from different sources such as waste to obtain goods and services.

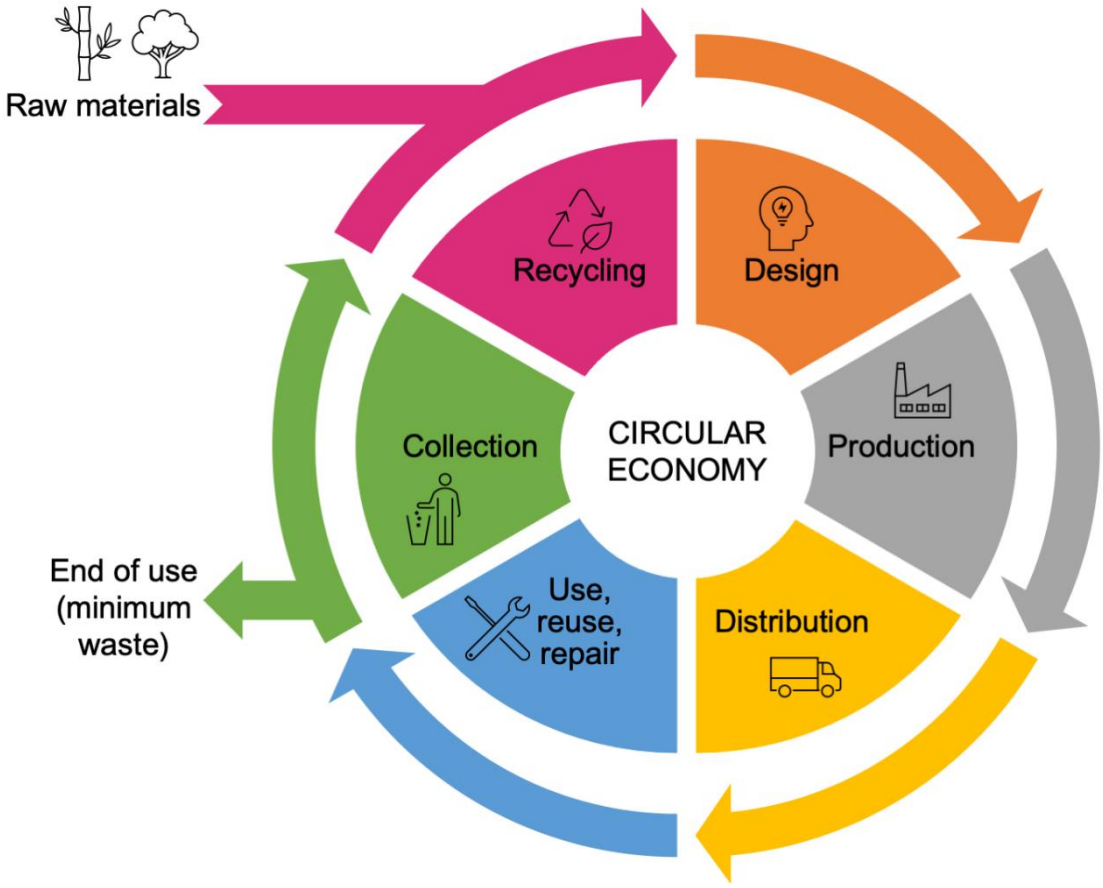

Figure 2.1. The steps that shape a circular economy. Adapted from European Parliament (European Parliament, 2015).

The products (chemicals, energy, etc) from biorefineries can be classified according to the substrate used. Regarding this, the origin of the biomasses assigns them into generations. First-generation biorefineries employ food-based crops rich in sugar or starch components such as sugarcane, corn, or molasses. These feedstocks are easy to be economically competitive, nevertheless they carry the problem of competing with the food supply chain (Satari et al., 2019). One of the issues with first-generation chemicals is the indirect land use change. This refers to the shifting of the use of the pasture or agricultural land destined for food production to mainly biofuel/chemical production. However, the demand of food is steady which may require the utilization of new land with high carbon stock, such as, forest or wetlands. In the end it would lead to an increase in the GHG emissions. In order to reduce the impact on the food supply chain, in 2015 the European Union limited the use of cereal and other starchy rich crops for energy purposes, such as, liquid biofuels in the Directive 2015/1513 (The European Parliament and the Council of the European Union, 2015).

Second-generation biorefineries employ non-food-competitive residues which overcomes the drawback of the production of first-generation biofuels. These residues are mainly originated from agricultural or forestry activities but can also use other industrial waste such as food waste, glycerol, etc. Among them, the major feedstock is the lignocellulosic waste which is abundant, sustainable, and low-cost. Lignocellulosic waste may come from agriculture (stoves, bagasses or straws), forestry (softwoods and hardwoods) or industries (paper mill discards, etc.). The main technical-economic bottleneck on the use of lignocellulosic feedstocks lies on their recalcitrant structure requiring an expensive pretreatment to separate its components (Naik et al., 2010; Satari et al., 2019). The third-generation biorefineries employ macro or microalgae, while the fourth-generation use engineered algae from oxygenic photosynthetic organism (Satari et al., 2019). The advantages of the employment of algal biomass are the fast growth compared to land plants, the lacks of competition for agricultural land and their capture of CO_2. However, the main drawbacks are their harvesting of either the macro or microalgae, along with the need for carbon supply (Ramachandra and Hebbale, 2020; Satari et al., 2019). This PhD thesis will be focused on the exploitation of lignocellulosic waste to produce second-generation bioalcohols.

2.2. Biorefineries for bioalcohols production

The bioalcohol demand is increasing globally due to their wide applications in markets, such as, energy or manufacturing. In 2019 the global market of industrial alcohol was 111.26 billion USD and is projected to exhibit a compound annual growth rate of 11.7% during the 2019-2027 period (Fortune Business Insights, 2020). Table 2.1 introduces the uses and production routes (chemical synthesis and fermentative pathways) of some bioalcohols of interest, such as, bioethanol, biobutanol and biohexanol. The chemical synthesis may require harsh conditions (temperature, pressure, etc) and several reactions in the case of butanol and hexanol (Table 2.1), whereas the fermentative production employ milder conditions over the chemical routes.

Table 2.1. Uses and production routes of bioalcohols of industrial interest (Baeyens et al., 2015; Dürre, 2008; Falbe et al., 2013; Fernández-Naveira et al., 2017a; Ibrahim et al., 2017; Panahi et al., 2022).

Chemical	Uses	Chemical synthesis	Main fermentation routes	Main microorganism
Ethanol	Biofuel; drug, plastic, or cosmetic industries	Acid-catalysed hydration of ethylene	Alcoholic fermentation	*Saccharomyces. cerevisiae, Saccharomyces tanninophilus; Zymomonas mobilis*
Butanol	Biofuel; production of butyl acrylate, methacrylate, plastics; industrial solvent; textile, and cosmetic industries	Petrochemically: oxo reaction from propylene, to butyraldehyde, which is hydrogenated to butanol	Acetone-Butanol-Ethanol (ABE) fermentation	*Clostridium acetobutylicum, Clostridium beijerinckii*
Hexanol	Biofuel; intermediates; plasticizer; lubricant	Ethylene to triethyl-aluminum followed by oxidation of the growth product, hydrolysis, and fractional distillation	Hexanol-Butanol-Ethanol (HBE) fermentation	*Clostridium carboxidivorans*

There are well-known applications for the bioalcohols. The ethanol is a chemical with a broad range of applicability with uses as biofuel, or in the production of drugs, plastic, plasticizers, and cosmetics among others (Table 2.1). Ethanol can be produced petrochemically by the hydration of ethylene with an acid catalyst. However, the fermentation pathway is used for the production of biofuel grade bioethanol and alcoholic beverages. The most used microorganisms for ethanol production are *S. cerevisiae* and *Z. mobilis* (Baeyens et al., 2015; Devi et al., 2022).

The butanol can be employed as a biofuel, as a chemical intermediate to produce butyl acrylate, methacrylate and plastics or as a solvent to produce glycol ethers, butyl acetate, antibiotics, vitamins or hormones. Butanol is also used as a swelling agent for coating fabric in textiles and in cosmetic industries (Moon et al., 2016). Butanol is currently produced petrochemically but can also be produced biologically as is the major product of the ABE fermentation via bacteria from the genus *Clostridium* (Table 2.1). The hexanol is utilized as biofuel, plasticiser, lubricant, or chemical intermediate. The

chemical synthesis of hexanol requires a series of stages while it can also be produced via the HBE fermentation with *C. carboxidivorans* (Table 2.1). Among the bioalcohols of industrial interest, this PhD thesis is focused on the development of platforms based on *Clostridium* spp. for the production of biobutanol and biohexanol using lignocellulosic feedstocks.

Due to the high versatility of bioalcohols, several industries have tried to develop economically feasible processes to their production by *Clostridium* fermentation or other microbial species. The production of second-generation bioethanol has been industrially implemented by the company LanzaTech in several locations in Asia and Europe. This company has developed a continuous industrial process with *Clostridium autoethanogenum* that uses waste gas streams from steel industries or biomass gasification in order to produce bioethanol (Fackler et al., 2021). Nevertheless, the importance of these bacteria resides on their production of higher carbon chained bioalcohols. For instance, the company Green Biologics developed a first-generation biobutanol platform from a modified *Clostridium* species, however the activity of the company was terminated in 2019 (Vees et al., 2020). Another example of industrial bioalcohol production is the USA-based company Gevo which is currently producing isobutanol from a modified yeast as first-generation biofuel from corn starch (Veza et al., 2021).

2.2.1. Bioalcohols to decarbonise the transport sector

The main interest of bioalcohols lie on their use as biofuels which would help to achieve a carbon neutral economy. According to the IEA, the global demand of biofuels in 2021 was 157 billion litters with an annual average increase of 4% up to 2026 reaching a forecasted demand of 186 billion litres. Among the bioalcohols, bioethanol has the highest demand of biofuel in the planet (>50%). This demand is expected to increase the highest among biofuels up to 2026, in which India would rise to the third position on ethanol consumption (IEA, 2021). The vast majority of bioethanol (80%) used for biofuels is produced in USA and Brazil from corn and sugarcane feedstock as a first-generation biofuel (Baeyens et al., 2015; Devi et al., 2022). The flex-fuel group of cars can use gasoline, pure ethanol or a mixture. For example, currently more than 20% of Brazil's fleet of cars can utilize 100% ethanol in ethanol-only and flex-fuel engines. In the USA, flex-fuel vehicles can use a range of 0-85% ethanol. Remarkably, global governments are regulating the proportion of biofuel blending in order to increase the use of biofuels and to reduce the effect of climate change (Baeyens et al., 2015; IEA, 2021).

Bioethanol can be used as a substitute of gasoline but is incompatible with diesel engines. There is a rising interest in higher carbon chain alcohols (such as butanol and hexanol) for its use as blends in either gasoline or diesel engines. The biobutanol has a series of advantages that facilitate its implementation on the transport sector. Butanol is less corrosive and it can transported and delivered with the existing pipelines and gas stations which would reduce the cost of infrastructures for the transition to a greener economy (Moon et al., 2016). Biobutanol can be used directly in current gasoline engines with a pure blend or in any proportions with gasoline. Moreover, biobutanol can also be employed up to 40% in volume with diesel blends. Also, hexanol is an attractive biofuel with favourable aspects which has been blended up to a 30% in diesel engines (De Poures et al., 2017). The different fuel properties of diesel and butanol and hexanol are indicated in Table 2.2. The increment on the number of carbons augments the cetane number, low heating value, density, viscosity, and flash point. Contrarily, it decreases the oxygen content, volatility, vapour pressure and solubility in water. Apart from the use of bioalcohols in road traffic engines, there is a growing attention on exploring the use of bioalcohols as jet fuel. Bioethanol, biobutanol or other longer carbon chain bioalcohols can be converted to jet synthesized paraffinic kerosene which can be blended up to a 50% in jet engines (Kurzawska and Jasiński, 2021).

Table 2.2. Fuel properties comparison between diesel and bioalcohols that can be blended with diesel. Adapted from De Poures et al. (2017).

Properties	**Diesel**	**1-Butanol**	**1-Hexanol**
Molecular formula	C_xH_y	C_4H_9-OH	C_6H_{13}-OH
Molecular weight (kg kmol^{-1})	190-211.7	74.1	102.2
O (wt.%)	0.0	21.6	15.7
Cetane number	>47	17	23
Lower heating value (MJ kg^{-1})	~42.5	33.1	39.1
Latent heat of evaporation (kJ kg^{-1})	<300	708	603
Vapour pressure (mm Hg)	0.4	7.0	1.0
Density at 15 ºC (kg m^{-3})	835.0	809.7	821.8
Viscosity at 25 ºC (mPa s)	2.72	2.54	3.32
Solubility in water (g L^{-1})	Immiscible	77.0	7.9
Self-ignition temperature (ºC)	~300	345	285
Boiling point (ºC)	125-400	118	157
Flash point (ºC)	>55	35-37	59

2.3. Lignocellulosic feedstocks to produce second-generation bioalcohols

2.3.1. Composition of lignocellulosic feedstocks

The lignocellulosic feedstocks are vegetal materials mainly composed by cellulose, hemicellulose and lignin alongside with other minor components such as pectin, extractives, ashes and proteins. A simplified conceptualization of the structure of lignocellulosic biomasses is depicted in Figure 2.2. The principal component of the lignocellulosic biomass is the cellulose, an homopolysaccharide composed of β-D-glucopyranose monomers (joined by β-1,4-glycosidic bonds). The glucose monomers form vast chains with a degree of polymerization up to 14000 units (Satari et al., 2019). These chain formations gather to form nano- and micro-fibrils joined by inter- and intra-molecular hydrogen bonds due to the presence of hydroxylic groups. Conditioned by the orientation of the cellulose molecules two regions can be observed, crystalline or amorphous regions. The amorphous region is easier to hydrolyse into its monomers (Mirmohamadsadeghi et al., 2021).

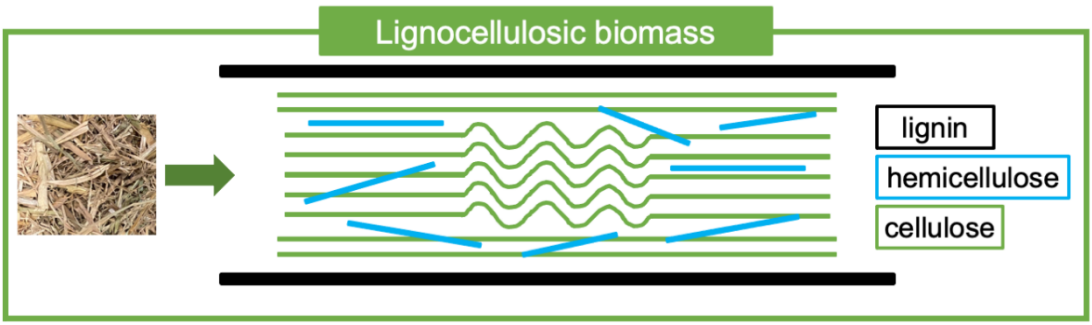

Figure 2.2. Conceptualization of the structure of lignocellulosic biomass.

The secondary polysaccharide of the lignocellulose structure is the hemicellulose. This heteropolysaccharide has a branched and amorphous structure composed by pentoses (D-xylose, L-arabinose, or L-rhamnose) and hexoses (D-glucose, D-galactose, or D-mannose). The monosaccharides are joined by β-1,4-glycosidic bonds with a degree of polymerization around 200 units. The hemicellulose core and branch-chain composition along the degree of polymerisation can vary to the plant species and the cell type (Kumar et al., 2022). The main component of the hemicellulose is the xylan which the major monomer in composition is the D-xylose. Due to the prevalence of this pentose in the hemicellulose structure, the D-xylose is the secondary monosaccharide in composition of lignocellulosic biomasses after glucose.

Moreover, the D-xylose is normally substituted with acetyl groups. The hemicellulose is also composed by other elements such as acids (galacturonic acid, methyl glucuronic acid or glucuronic acid). The structure of hemicellulose is more prone to decay than that of cellulose (Satari et al., 2019).

Lignin is a three-dimensional polymer composed of phenylpropane units (coniferyl alcohol, coumaryl alcohol and sinapyl alcohol). The monomers are linked by carbon-carbon and ester bonds forming a hydrophobic and rigid structure (Tan et al., 2021). The degree of polymerization ranges from 450 to 550 units. The lignin acts as bonding agent to hold the components into a recalcitrant structure that increases the integrity of the biomass (Figure 2.2). Cellulose, hemicellulose and lignin quantify over the 80% of the total mass of the lignocellulosic biomasses. The remaining percentage includes heterogeneous materials divided into extractives (waxes, fats, phenols, etc.) and nonextractives (silica, oxalates, etc.) (Mirmohamadsadeghi et al., 2021). The natural recalcitrant structure of the lignocellulosic biomass makes difficult the cleavage of their components into fermentation substrates, being this step the main bottleneck for its usage. Hence preliminary treatment prior microbial fermentation is required (Kumar et al., 2022; Mirmohamadsadeghi et al., 2021).

2.3.2. Paddy rice as a source of lignocellulosic waste

Lignocellulosic waste comprises a broad range of materials to be used in the production of second-generation bioalcohols. Agricultural waste, forestry waste, industrial waste or municipal solid waste are common sources of lignocellulosic feedstocks. Among them, the utilization of agricultural waste with less percentage of lignin compared to softwood or hardwoods may increase the techno-economic feasibility of producing bioalcohols via fermentation. Moreover, the widespread distribution of crops and their huge waste production results in a great potential source of raw lignocellulosic materials to be processed into bioalcohols. The key points for achieving sustainable biorefineries are the composition of the lignocellulosic waste and its local availability. Regarding the waste composition, microbial fermentations are directly influenced to the monomeric sugar release that can be estimated from the cellulose and hemicellulose content. Satlewal et al. (2017) reviewed the characteristics and composition of rice straw waste which showed a chemical variability depending on their origin. Table 2.3 compiled the components and its weight ranges. Regarding the local accessibility, in 2021 rice was the third most harvested crop in the planet in terms of cultivated area after the wheat and maize. The principal production is located in Asia: India (28%), China (18%), Bangladesh and Thailand (7%, each one) and Indonesia (6%). In Europe, Spain is the

third producing country with a 14% of the continental production after Italy (37%) and the Russian Federation (31%) (FAO, 2021). Moreover, rice is an agricultural crop of regional interest in the Valencian Community, cultivated mainly in the Albufera National Park. This landmark is one of the most important and representative wetlands from the Mediterranean basin and it is located at 10 km of the metropolitan area of Valencia. During 2021, a total of 15,282 hectares were used for the cultivation of paddy rice in this National Park. This extension represented 18% of the total area harvested in Spain (Conselleria de Agricultura Desarrollo Rural Ecológica Emergencia Climática y Transición, 2021). The local and wide availability of rice straw was the attractive to use this feedstock as raw material in this PhD thesis.

Table 2.3. Chemical composition of rice straw. Adapted from Satlewal et al. (2017).

Component	Quantity (% wt)
Glucose	34.0-43.7
Xylose	19.0-22.0
Arabinose	2.0-3.6
Mannose	1.8-2.0
Acid soluble lignin	2.2-6.0
Acid insoluble lignin	13.0-22.7
Ash and silica	7.8-20.3
Extractives	16.1-19.3

The paddy rice generates two main lignocellulosic wastes, rice straw and rice husk. After harvesting the rice grain, the rice straw remains in the open field. The rice straw production can be estimated by applying a factor 1.5 to the gross rice production (Gadde et al., 2009). Traditionally, the management of rice straw was done by on-field burning as it is the fastest and cheapest way to destroy it. However, this strategy causes air pollution by particulate matter emissions to the surrounding areas and nutrient loss of N, P, K and S (Domínguez-Escribà and Porcar, 2010). Currently, this procedure is forbidden in the European Union. Alternatively, the recycling of rice straw into the soil by its on-field grinding can regain the nutrients. However, this management alternative implies its on-site anaerobic digestion, resulting in fugitive emissions of CH_4 that should be avoided due to its climate forcer properties. It also could lead to putrefaction resulting with the ictiofauna mortality (Domínguez-Escribà and Porcar, 2010). Nowadays, the Valencian Government is implementing active policies to collect the rice straw in L'Albufera National Park in order to use it for animal feeding or bedding, mulching, green building and compost, among others (Conselleria de Agricultura, Desarrollo Rural, 2022). However, one of the drawbacks of the *ex situ* utilization of the rice straw is its light density which difficulties the transportation to the processing facility (Domínguez-Escribà

and Porcar, 2010). Following these policies, new alternatives for the rice straw management are required. In this regard, the use of rice straw for the production of second-generation bioalcohols is a very interesting option due its high monosaccharide and moderate lignin contents (Vivek et al., 2019).

2.3.3. Processing of lignocellulosic waste

The valorisation of lignocellulosic feedstocks requires its pre-processing prior to microbial utilization. The most common pre-processing procedure aims to release the fermentable sugars. This process is usually carried out in two steps: a tailor-made pretreatment to break down the lignocellulosic structure followed by a hydrolysis step to release the monosaccharides. Due to the high cost of the conventional pre-processing scheme, novelty routes of processing lignocellulosic feedstocks are nowadays under research. For example, the gasification of the lignocellulosic waste into a syngas stream and its subsequent fermentation is seen as a potential alternative to produce bioalcohols via biorefinery.

2.3.3.1. Monosaccharide release by pretreatment

Most microorganisms are capable of uptake monosaccharides to grow and develop. These monosaccharides can be obtained from the cellulose (glucose) and hemicellulose (xylose). Nevertheless, the recalcitrant structure of the lignocellulosic feedstock difficulties the easy release of the sugar monomers. The accessibility of the cellulose and hemicellulose can be promoted by using a pretreatment for breaking the hydrogen bonds. The altering of the intermolecular bonds leads to a boost in the amorphous structure, porosity, and surface area, thus, facilitating the action of the hydrolytic enzymes to release the monosaccharides (Kim, 2018). The pretreatment of lignocellulosic biomass may start with the milling and sieving to a lower particle size to increase the efficiency of the process. Figure 2.3 depicts the overall process for the production of bioalcohols from lignocellulosic feedstocks. The pretreatment process results into two phases (solid and liquid). The liquid fraction is enriched in lignin and hemicelulose while the solid fraction is enriched in cellulose. The solid fraction usually requires a subsequent enzymatic hydrolysis to release the monomeric sugar content. The hydrolytic cocktail of enzymes is composed of cellulases and hemicellulases. The cellulases can be endo-1,4-β-glucanases, which randomly cuts the internal bonds of cellulose; exo-1,4-β-D-glucanases, which attacks the ends of celluloses and 1,4-β-D-glucosidases that hydrolyse cellobiose and cellodextrins into glucose. Whereas, the hemicellulases are a pool of enzymes that synergistically hydrolyse hemicellulose.

These enzymes can be divided in two groups: depolymerases and debranching enzymes. The depolymerases attack either internally (*endo*) or the ends (*exo*) of the chains which can be xylanases, β-glucanases or manases among others. Meanwhile, the debranching enzymes can be α-glucuronidases, α -arabinofuranosidases or acetyl xylan esterase and suchlike. The enzymatic hydrolysis is influenced by the enzymes themselves, but also by the physicochemical and morphological characteristics of the biomass (lignin content, morphology of the polysaccharides, presence of acetyl groups, etc.). Moreover, the remaining lignin fraction could impact negatively as it can hinder the enzymatic hydrolysis by either enzymatic adsorption to the solid lignin; physical blocking of the accessibility to the polysaccharides or enzyme deactivation by soluble lignin compounds (Satari et al., 2019).

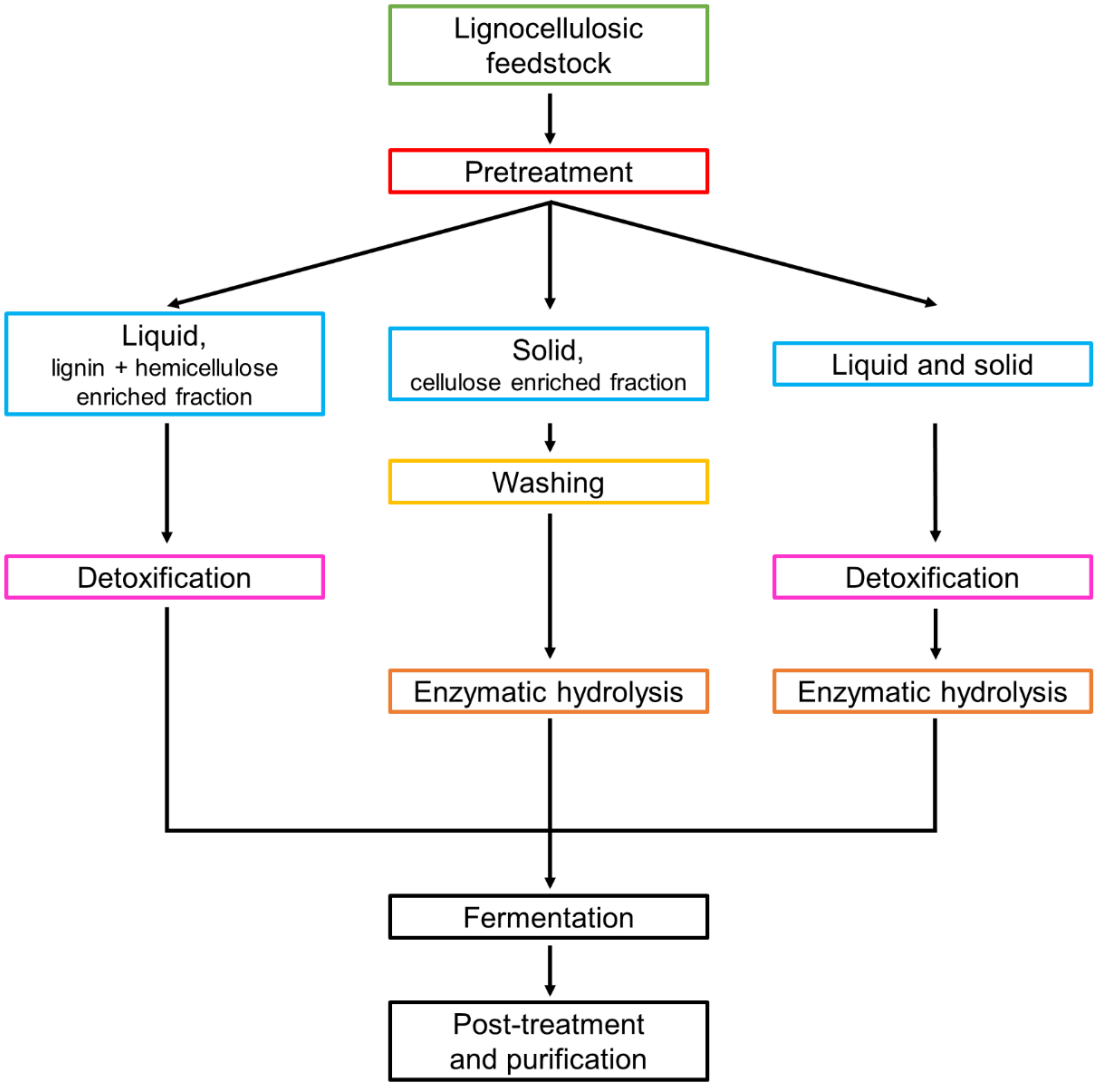

Figure 2.3. Overall scheme for the production of bioalcohols from lignocellulosic feedstocks using monosaccharide release by pretreatment. Adapted from Amiri and Karimi (2018).

The cleavage of the lignocellulosic feedstocks during the pretreatment and posterior hydrolysis step may produce a broad range of inhibitory compounds to microorganisms. The formation of these toxic compounds is highly related to the biomass composition and the pretreatment utilized (Satari et al., 2019). These compounds can be classified according to their functional groups into sugar-derived aldehydes; short-chain organic acids and aldehydes; aromatic compounds, and other inhibitors, as metals. Inhibitory chemicals can adversely impact on the performance of the enzymatic hydrolysis and/or the posterior fermentation. Among the sugar-derived aldehydes, it can be pointed out the 5-hydroxymethylfurfural (5-HMF) and furfural from the degradation of the hexoses and pentoses, respectively. Both molecules do not hinder the hydrolysis step but the microbial growth. The most important short chain molecules produced are acetic acid derived from the acetyl groups; formic acid derived from the degradation of furfural and 5-HMF or levulinic acid derived from the degradation of 5-HMF. These weak acids have a negative impact on the development of the microbial fermentation instead of the hydrolysis step itself. Meanwhile, aromatic compounds are classified into phenolic compounds, non-phenolic compounds and benzoquinones which mainly originates from the lignin portion of the feedstock. Aromatic inhibitors negatively influence not only microorganisms but also to the hydrolytic enzymes (Sjulander and Kikas, 2020).

The production of bioalcohols via microbial fermentation is interfered by the possible inhibitors obtained during the pretreatment and hydrolysis step. Hence, a detoxification step to remove them may be necessary. The detoxification procedures can be performed by several techniques that range from the chemical degradation (e.g., overliming); adsorption (e.g., activated charcoal); extraction (e.g., liquid-liquid extraction) or filtration (e.g., nanofiltration). Some microorganisms have developed natural tolerance to inhibitors such as, the production of detoxifying enzymes (Sjulander and Kikas, 2020). To remove the inhibitors or the rest of chemicals used in the pretreatments the solid fraction from the pretreatment step can be easily washed (Figure 2.3). This PhD thesis will be focused on the delignified solid fraction; the liquid fraction will be discarded as it contains as major component the solubilized lignin that will inhibit the further fermentation process.

Regarding the pretreatment process, it should be effective in a wide range of biomasses with different characteristics. It should increase the action of the hydrolytic enzymes while solubilizing hemicellulose and/or lignin. The ideal pretreatment would result in low sugar loss and little production of sugar degradation products or inhibitory compounds. Moreover, the high cost related to energy consumption or chemical dosing

remains as one of the main drawbacks to develop lignocellulosic biorefineries. Several pretreatments as physical (e.g., microwaves), chemical (e.g., organosolv, acid, alkaline or ionic liquids), physicochemical (e.g., steam explosion or ammonia fibre expansion) or biological procedures have been explored. Table 2.4 summarizes main advantages and disadvantages from different pretreatments for the lignocellulosic feedstocks.

The microwave-assisted pretreatment is a purely physical process. It acts over the biomass in short reaction times by uniformly heating causing a quickly solubilisation of the hemicellulose and lignin. The drawback of this technology is the high investment in specific machinery and the overall process cost. From the previous work in our research team, Valles et al. (2020) recovered an 80.5% of the original rice straw when a microwave-assisted hydrothermolysis was carried out. The operation conditions were a 10% (w/v) solid loading in a 1,500 W microwave at 200 ºC for 15 min. This procedure provoked losses of a 11.5%, 50.6% and 34.5% for glucan, arabinan and xylan, respectively. These results clearly indicate the high effectiveness on removing the hemicellulose. Concomitantly, a 13.3% delignification was obtained which improved the posterior enzymatic hydrolysis step of the pretreated rice straw.

Several chemical compounds can be employed to alter the lignocellulosic structure such as, acids, alkalis, solvents or ionic liquids in different concentrations and temperatures. The use of acid and alkalis as pretreatments is widely utilized with lignocellulos biomasses (Tan et al., 2021). The acid pretreatment, especially with the use of H_2SO_4, highly hydrolyses the hemicellulose fraction of the feedstocks increasing the accessibility to cellulose by the hydrolytic enzymes. This pretreatment can be performed in high acid concentration with low temperature and vice versa. The first one is more economically viable but increases the production of inhibitors and the degradation of monosaccharides. Whereas the use of diluted acid concentrations is more attractive due to the lower inhibitors production and the better recovery of the hemicellulose monosaccharides in the liquid fraction of the pretreatment (Mood et al., 2013). For example, Hsu et al. (2010) obtained a maximum sugar yield of 83% when rice straw was pretreated with 1% H_2SO_4 (w/w) with a reaction time of 1-5 minutes at 160 or 180 ºC. In this study, it was also observed that the lignin content and the crystallinity of the solid fraction did not clearly relate to the yield of the subsequent enzymatic hydrolysis. Therefore, it seems that the lignin remaining on the pretreated feedstock under these conditions would not be a key factor of the hydrolysis step.

Table 2.4. Advantages and disadvantages of several procedures for the pretreatment of lignocellulosic feedstocks. Adapted from Ahmad et al. (2020); Amiri et al. (2014); Baeyens et al. (2015); Kim, (2018); Mood et al. (2013); Sarkar et al. (2012); Singh et al. (2016); Vivek et al. (2019).

Pretreatment procedure	Type	Advantages	Disadvantages
Microwave-assisted	Physical	Uniform and fast heating of the residue Short reaction times Good elimination of lignin and hemicellulose	Specific equipment required High energy consumption
Steam explosion	Physico-chemical	Lignin transformation and hemicellulose solubilisation Cost-effective Low energy requirement Null or little chemical utilisation	Production of inhibitory compounds Acid required with biomasses with high lignin content
Ammonia fibre explosion	Physico-chemical	Nitrogen source available for posterior fermentation High sugar recovery (>99%) Recycling of chemical Little production of inhibitors	Low effect on biomasses with high lignin content Low hemicellulose and lignin decay
Organosolv	Chemical	Lignin valorisation Hemicellulose solubilisation	High cost of chemicals Operating risk (volatility and flammability) Necessity of recovery and recycle of the solvent
Acid	Chemical	High glucose yield Hemicellulose solubilisation	Losses of sugars High cost of chemicals Specific equipment (corrosive resistant) Production of inhibitory compounds
Alkali	Chemical	Good elimination of lignin and hemicellulose Little production of inhibitors Reduction of polymerization degree and crystallinity of cellulose	Modification of lignin structure High use of water High cost of chemicals
Green solvent (Ionic liquids)	Chemical	Mild pretreatment conditions (low temperatures) Hemicellulose and lignin solubilisation High biomass loading	High cost of formulations Necessity of recovery and recycle of the solvent
Enzymatic	Biological	Mild conditions Little production of inhibitors Low energy requirement High sugar yield	High cost of enzymes Slow treatment Long procedure time

The alkali pretreatment with NaOH, KOH, NH_4OH, or CaOH is useful for the pretreatment of biomasses with low content in lignin such is the case of the rice straw. Main advantage of alkali pretreatment deals with the lower sugar degradation compared with the acid procedures. The alkali pretreatment attacks the lignin-polysaccharides formation by swelling its structure, thus it increases the internal surface area and reduces the crystallinity and polymerization degree of cellulose. The use of NaOH is sharply effective against the rice straw due to its effect on the structure of the silica layers and cuticle wax (Imman et al., 2015; Vivek et al., 2019). Previous research carried out in our research group was developed with alkali pretreatment for the exploitation of rice straw to biobutanol. These assays explored the effect of NaOH concentration (0-1% w/v) and reaction time (20-60 min) by using a five-level two-factor central composite design working at 134 ºC and 5% w/v of solid loading. Among both factors, the NaOH concentration resulted in stronger effect than the process time. The data modelling predicted a maximum butanol-biomass ratio when using 0.75% (w/v) NaOH and 20 min of pretreatment. The only noticeable inhibitory compound found from hydrolysis was acetic acid with undetected or very low concentrations of other inhibitors (levulinic acid, furfural, 5-HMF or phenolic compounds) (Valles et al., 2021b).

The organosolv pretreatment allows the latter purification of the extracted lignin increasing the potential exploitation of the lignocellulosic waste. An organosolv pretreatment of 75% (v/v) ethanol with 1% (w/w) of H_2SO_4 was performed with rice straw. The reactions were carried out with a 1:8 solid-to-liquid ratio at 150 or 180 ºC for 30 or 60 min. The increment of the temperature raised the lignin removal by >33% with identical time of pretreatment (60 min). This organosolv process partially dissolved the hemicellulose by 40-49%. The highest monosaccharide concentration in hydrolysates was obtained with the pretreatment at 150 ºC and 60 min. These conditions of pretreatment were not the best in terms of lignin removal (45%) over to the other conditions evaluated (60% lignin removal at 180 ºC and 60 min). Therefore, these results indicates that not only the lignin content in the solid phase is influencing the outcome of the enzymatic hydrolysis but also the severity of the pretreatment itself (Amiri et al., 2014).

The ionic liquids are organic salts with low melting points (<100 ºC) which have interesting properties such as low vapour pressures, elevated thermal and chemical stability, and recyclability. However, the high cost of ionic liquids could be a major drawback for their use as green solvent. Alternatively, other formulations of green solvents as deep eutectic solvents are seen as low-cost alternatives. Nowadays, it is

also raising of interest the use of biobased green solvents. In this case, natural deep eutectic solvents formulated with natural compounds are seen as greener alternatives (Douard et al., 2021; New et al., 2022) (Table 2.4). Our research group is currently working on the use of ionic liquids and deep eutectic solvents to pretreat the rice straw. For instance, the use of 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) was shown effective for pretreating rice straw at a solid loading of 5% (w/w). Among the tested operational conditions, the best conditions for temperature and reaction time were 120 ºC and 5 h. Best data resulted in a delignification of 65%. Regarding the inhibitory products on the hydrolysates, levulinic acid and furfural were not detected in any of the conditions tested from a central composite design of experiments (53-137 ºC; 1.5-6.2 h; 3.3-11.7% w/w solid loading), while acetic (<4.15 g L^{-1}) and formic (<1.13 g L^{-1}) acids were quantified (Poy et al., 2021).

There is a huge variability on the sugar concentration that has been employed in literature for microbial fermentations to obtain bioalcohols. Birgen et al. (2019) performed an exploratory review of the fermentative production of butanol using microorganisms of the genus *Clostridium* on batch reactors during the last three decades. This dataset enclosed 156 fermentations (79 synthetic mixed sugars and 77 lignocellulosic hydrolysates). This review showed that the median values for the fermentations with synthetic sugar media were 28, 23 and 60 g L^{-1} for glucose, xylose and total sugars, respectively, while for the fermentations with lignocellulosic hydrolysates were 23.6, 10.8, 41.8 g L^{-1}, for glucose, xylose and total sugars, respectively. These findings evidence that experiments performed with synthetic media tends to overestimate the concentrations of sugar release of lignocellulosic hydrolysates.

2.3.3.2. Gasification as an alternative for processing lignocellulosic waste

The gasification is a thermochemical process in which the organic solid biomass (food waste, lignocellulosic feedstocks…) is converted into gaseous products, mainly syngas. This syngas is a gaseous stream mostly composed by CO and H_2, but that can contain also CO_2, CH_4 or other minor gases. The biomass gasification process includes four main steps: drying, pyrolysis, combustion and reduction. A broad range of parameters such as the biomass composition, the percentage of moisture, the gasifying agent or the kind of gasifier reactor can modulate the composition of the resulted syngas (Mishra et al., 2023). The thermochemical treatments can be mainly classified into conventional or hydrothermal gasification, which differ in the composition of the gaseous products and their operational conditions. The conventional gasification is operated at

500-1400 ºC and atmospheric pressure with air, oxygen, CO_2 or steam as a gasifying agents. However, this process produces undesired by-products as chars, tars or nitrogen and sulphur impurities (Okolie et al., 2022). The hydrothermal gasification can be performed using supercritical water as gasifying agent. This treatment is proceeded above the critical point of water (>374ºC and >22.1 MPa), normally within a range of 350-650 ºC. This operation is suitable for biological feedstocks rich in moisture. The syngas from hydrothermal treatment does not contains nitrogen and sulphur impurities. Nevertheless, hydrothermal gasification is more expensive, and more susceptible to fire and/or explosion risks (Okolie et al., 2022, 2020). In this context, the conventional gasification would be more suitable for the lignocellulosic feedstocks as they have low water contents. In comparison with the conventional biomass pretreatment to release monosaccharides, gasification has advantage of nearly whole conversion of the biomass into syngas without noticeable carbon losses (Rathore and Singh, 2022).

The gasification of rice wastes is regarded as a promising technology to produce syngas. Rice straw gasification is scarcely reported compared to the other rice residue, the rice husk. Most studies use fixed bed reactors, either in downdraft or updraft configurations, although fluidized bed gasification and entrained bed reactors have also been employed (Dafiqurrohman et al., 2022). Syngas streams may further be either catalytically converted by the Fischer-Tropsch process into hydrocarbons or biologically treated by bacteria able to assimilate CO and H_2 into multi-carbon compounds (Rathore and Singh, 2022). Nowadays, the isolation of microorganisms capable of metabolizing syngas have encouraged research on C1 fermentation routes. Among syngas fermentation, some bacteria of the genus *Clostridium* have gained increased attention in recent years due to their ability to produce bioalcohols of industrial interest, such as, bioethanol, biobutanol or biohexanol (Fernández-Naveira et al., 2017a).

2.4. References

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3. *CLOSTRIDIUM* FERMENTATION PLATFORMS FOR BIOSOLVENT PRODUCTION

3.1. *Clostridium* fermentations

The microorganisms of the *Clostridium* genus are obligate fermentative anaerobes that employ a wide range of carbon sources such as, carbohydrates, carbon rich gases, purines, pyrimidines, or amino acids to grow. One of the most interesting groups of these bacteria are the solventogenic *Clostridium* spp., capable to produce solvents, such as, ethanol, acetone, butanol or hexanol. These solventogenic bacteria perform a biphasic fermentation to produce the solvents. Initially, on the acidogenic phase the bacteria uptakes the carbon source leading to an exponential growth of biomass coupled with volatile fatty acids and gas production alongside to a drop on the pH. The volatile fatty acid formation concomitant with the pH reduction leads to the solventogenic phase in which the microorganisms reassimilate the acid species, transforming them into solvents (Fernández-Naveira et al., 2017a). Among the solventogenic pathways, the ABE fermentation and the HBE fermentation are of interest due to their production of biobutanol and biohexanol.

3.2. ABE fermentation

Solventogenic *Clostridium* spp. are capable to grow on carbon sources (starch, glucose, xylose, inulin…) and produce solvents via the ABE fermentation, in which butanol is the main product of the fermentation. Among them, *C. acetobutylicum, C. beijerinckii* or *Clostridium saccharoperbutylacetonicum* are the most frequently studied (Patakova et al., 2013). Additionally, some *C. beijerinckii* strains perform a modified route where isopropanol is produced instead of acetone in the isopropanol-butanol-ethanol fermentation (Ahmed et al., 1988; Rochón et al., 2020). *C. acetobutylicum* was firstly isolated by Chaim Weizmann (US Patent 1,315,585) and was used in the World War I to obtain acetone and posterior production of powder cordite. Between 1920 and 1960, butanol was industrially obtained from carbohydrate-rich carbon sources (molasses, grain, etc.) via ABE fermentation. However, the low cost of petroleum circa the 1950s shifted the industrial production to the petrochemical way. Currently, there is a renovated interest on this process as greener options are required to obtain butanol (Pugazhendhi et al., 2019). Figure 3.1 depicts the metabolic pathway of the ABE fermentation from *C. acetobutylicum*, which is the selected bacteria for this PhD thesis. After the uptake of the monosaccharides by the cell, the hexoses are assimilated via the Embden-Meyerhof-Parnas (EMP) (Figure 3.1, black box) and the pentoses can be either metabolised via the pentose phosphate pathway (PPP) (Figure 3.1, green box) or the phosphoketolase pathway (PKP) (Figure 3.1, yellow box) (Liu et al., 2012; Vees et al., 2020). During the acidogenic phase, *C. acetobutylicum* consumes the monosaccharides producing acetic

and butyric acids alongside with CO_2 and H_2. Later on, during the solventogenesis the acetic and butyric acids will be reassimilated and converted into the ABE products (Monot et al., 1982).

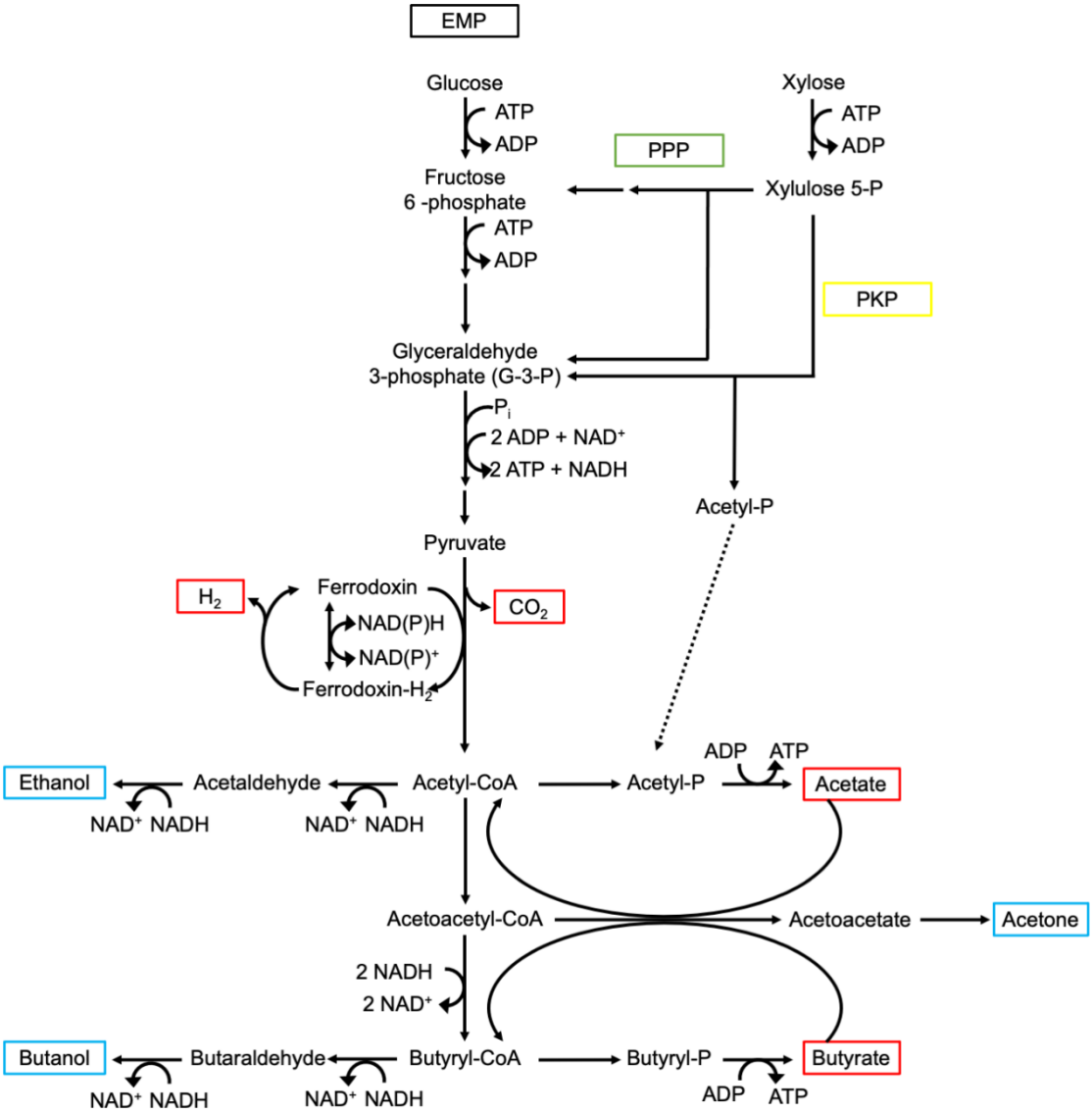

Figure 3.1. Scheme of the metabolism of the ABE fermentation by *C. acetobutylicum.* EMP pathway for glucose (black box), PPP (green box) and PKP (yellow box) for xylose. Red box: products of acidogenesis. Blue box: products of solventogenesis. The metabolism of glucose produces 2 G-3-P molecules, the following reactions into the pathway refers to the metabolism of one just G-3-P. Adapted from Liu et al. (2012) and Moon et al. (2016).

3.2.1. Factors affecting ABE fermentation

There are several factors that influence the outcome of the ABE fermentation. For instance, the behaviour of microorganisms can be modulated by external conditions (temperature, agitation, etc.) or suffer from substrate/product inhibition disrupting the development of the fermentations (Ibrahim et al., 2017; Li et al., 2022). Particularly, the influence of pH and carbon source are among the most important factors in the development of ABE fermentation. Next, in this section both factors are described as they are the object in this PhD thesis. Also, the product toxicity is also discussed to show the limits of the ABE fermentation.

3.2.1.1. The pH

In the ABE fermentation, the pH plays a key role because it can modify the outcome of the process even by changing the metabolic route. The bacteria of the genus *Clostridium* exhibit a range of optimum pHs from around 4.5 to 6.0 depending on the bacterial strain and the product of interest (Buendia-Kandia et al., 2018; Gao et al., 2016; M. Jiang et al., 2014; Yang et al., 2013). An optimum pH for biomass development favours the acidogenesis phase leading to a higher acid production in detriment of solvent production. The decrease of the pH to a value close or lower than 5.0 increases the solvent production alongside with the reassimilation of the acids previously produced (Raganati et al., 2022). This is related to the stress environment provoked by the volatile fatty acid concentration and the pH reduction in the media. The bacteria are capable of maintaining their intracellular pH by several mechanisms of pH homeostasis, being one of them the conversion of acids into solvents (Abubackar et al., 2018).

The pH also determines the form of the volatile fatty acids that appears in dissolution and it has a directly impact on the metabolism of the microorganisms. The acetic and butyric acids produced by the bacteria can be found in the fermentation media as two species: the undissociated and the dissociated forms. These forms are directly related to the specific pK_a of each volatile fatty acid in which higher pH in the culture results in higher proportion of the dissociated form. The dissociated form, due to its negative charge, cannot permeate the cell membrane of the bacteria while the undissociated form can freely permeate the membrane. Once entered the cell, the undissociated acid is deprotonated due the higher intracellular pH, which can lead to an acidification of the cytoplasm. For example, *C. acetobutylicum* is able to maintain an internal pH of ~6.0 when the external pH diminishes up to 4.5 due to its deacidification systems (Huang et al., 1985). However, the over acidification of the internal pH can lead

to an imbalance of the proton exporting capacity. Under this condition, the fermentation process may end up in an acid crash phenomenon, stopping the cell growth, the monosaccharide consumption and the solvent production. Hence, the combination of an overproduction of acids and low pHs in the fermentation broth may result in adverse conditions if uncontrolled. The threshold of undissociated acid concentration in the fermentation media that causes acid crash varies within the microorganisms. For instance, Maddox et al. (2000) set a threshold value 57-60 mM of total undissociated acid that would cause the acid crash with *C. beijerinckii* NRRL B592.

The increment of the solvent production and productivity can be accomplished by effectively regulation the pH of the fermentation broth and thus the concentration of undissociated acids. Traditionally, the regulation of the pH has been carried out by buffering the fermentation media with several compounds, such as, ammonium acetate, calcium carbonate or phosphate species (Gottumukkala et al., 2013; Monot et al., 1982; Raganati et al., 2015; Yang et al., 2013). Alternatively, more sophisticated strategies such pH control has been developed to modulate the pH via chemical species (NaOH, CaOH, HCl, NH_4^+…) by setting a pH set-point at which the fermentation will be conducted (Buendia-Kandia et al., 2018; Luo et al., 2019; Raganati et al., 2016). Regarding this, M. Jiang et al. (2014) screened the effect of a range of pH values from 4.9 to 6.0 by controlling the pH with HCl or NaOH. Among the pH set-points evaluated, the value of 5.5 was found best for butanol production and productivity with *C. beijerinckii* IB4. The chemical control of pH can also be carried out to shift the fermentation pathways resulting in acids rather than solvents. Buendia-Kandia et al. (2018) used a pH set-point of 5.5 to favour acidogenesis with almost residual solvent production with *C. acetobutylicum* ATCC 824. Moreover, the differences of the fermentation outcomes with different microbial strains at identical pH set-point of 5.5 indicates the importance of selecting the optimum pH control for each fermentative target.

The effect of pH control for ABE production using rice straw hydrolysates, the lignocellulosic feedstock employed on this PhD thesis, has not been studied until now. Nevertheless, the beneficial effect of the chemical pH control was explored on corn stover hydrolysates. Su et al. (2022) better exploited a detoxified corn stover hydrolysate by the implementation of a pH at 6.5 during the initial 12 h of fermentation. This pH modulation incremented the monosaccharide consumption from 15.18 g L^{-1} to complete depletion (~40 g L^{-1}) with *C. acetobutylicum* ABE-P. The pH control avoided the toxicity of the corn stover hydrolysate resulting in an ABE production of 14.07 g L^{-1} over the 3.66 g L^{-1} achieved with a reactor with no chemical pH control. The results herein clearly

remark the importance of looking for strategies of pH regulation that maximizes the performance of ABE fermentation with *Clostridium* spp. to produce bioalcohols.

3.2.1.2. Carbon source

The carbon source is another of the key parameters that modulates the fermentation outcomes. In this regard, the use of lignocellulosic feedstock arises diverse challenges to better exploit its resources, such as the presence of monosaccharide mixtures or potential inhibitors. Microorganisms metabolize glucose over any other monosaccharide, including the xylose, the secondary monosaccharide in composition of lignocellulosic waste. This preference is referred as the Carbon Catabolite Repression (CCR) in which the presence of glucose strongly incapacitates the metabolism of other monosaccharides such as xylose or mannose. Thus, the CCR hinders the performance of the fermentations and reduces the butanol yield. The CCR can occur differently on the bacterial species. For instance, *C. beijerinckii* can consume xylose easily due to it has a gene cluster comprising most of the genes related to the pentose metabolism. However, *C. acetobutylicum* has the genes scattered throughout its chromosome which difficulties the xylose degradation (Gu et al., 2010).

Two alternatives to reduce the effect of CCR and to enhance the usage of xylose from lignocellulosic hydrolysates are under study. The genetic modification of the microorganisms has provided alternatives for the xylose conversion in presence of glucose with *Clostridium* spp. For example, Fu et al. (2022) double deactivated the genes *hprK* and *xylR* obtaining a *Clostridium tyrobutyricum* strain capable of metabolize glucose and xylose concomitantly, hence eliminating the CCR. In the case of *C. acetobutylicum*, the disruption of the *ccpA* gene, encoding a pleiotropic regulator (CcpA), allowed a simultaneous consumption of glucose and xylose without CCR (Ren et al., 2010). Moreover, Xiao et al. (2011) obtained a *C. acetobutylicum* strain capable of efficiently co-ferment glucose, xylose, and arabinose by the disruption the *glcG* gene and the overexpression of genes from the PPP (*xylT*, *xylA*, and *xylB*).

The other alternative is searching operational strategies to improve the xylose conversion. The xylose metabolism is possible when used as sole monosaccharide in *C. acetobutylicum* with different strategies to regulate the pH. (Hu et al., 2011; Kudahettige-Nilsson et al., 2015; Raganati et al., 2015). Indeed, Liu et al. (2012) observed that the increment of the xylose concentration from 10 to 20 g L^{-1} raised the degree of total xylose utilization from a 15 to 40% by the PKP. This could be related to a rate-limiting step in the PPP which would favour the PKP regardless its poorer energetic yields. However,

the fermentation of monosaccharide mixtures with *C. acetobutylicum* is strongly difficult and the glucose:xylose ratio can also influence the outcome of the fermentation profiles. Besides, the pH control has been indicated to improve the xylose consumption in presence of glucose despite its monosaccharide ratio (W. Jiang et al., 2014).

3.2.1.3. Product inhibition

Despite not being in the scope of this PhD thesis, the product inhibition is also introduced due to its importance when employing monosaccharide mixtures in the ABE fermentation. Among ABE solvents, butanol has the highest toxicity with a threshold of <2% wt. for the solventogenic *Clostridium* spp. Indeed, the butanol concentration affects differently to *C. acetobutylicum* depending on the monosaccharide utilized. Specifically, the inhibitory concentration of butanol when grown with glucose is over 13-14 g L^{-1} while with xylose is close to 8.5 g L^{-1} (Ounine et al., 1985; Raganati et al., 2015). Due its lipophilic characteristics butanol disrupts the phospholipids components of the cell membrane increasing its fluidity. This change in the properties may end in the destabilization of the cell membrane and the interruption of the normal membrane activities as the transport of molecules or the ATPs activity (S. Y. Lee et al., 2008). In any case, butanol inhibition causes low solvent titers, and restricts the monosaccharide concentration to be employed.

The inherent solvent toxicity may be alleviated by removing the butanol from the fermentation broth. The coupling of *in situ* product recovery (ISPR) techniques to the fermenter can decrease the solvent concentration in the fermentation broth, thus lengthening the fermentation time and increasing the amount of carbon source metabolized and the overall solvent production. The ISPR techniques for selective butanol separation must fulfil several requisites as low cost, biocompatibility, high affinity, easy and full regeneration, robustness and modularity. The ISPR can be classified into solvent- (liquid-liquid extraction, perstraction, etc.), absorbent- (packed bed, etc.) or vapour- (pervaporation, gas stripping, etc.) based technologies (Staggs and Nielsen, 2015). Gas stripping, vacuum distillation, flash fermentation and pervaporation are the most suitable candidates for industrial scale-up (Jiménez-Bonilla and Wang, 2018). Several ISPR techniques have been applied in ABE fermentation, such as gas stripping (Rochón et al., 2017; Valles et al., 2021a), pervaporation (Van Hecke et al., 2017), liquid-liquid extraction (Survase et al., 2019) or adsorption (Raganati et al., 2022).

3.2.2. Fermentation modes for butanol production using lignocellulosic feedstocks

The ABE fermentation can be carried out in batch and continuous modes. The operation mode determines the total production and productivity. In the following section is summarized the development of batch and continuous operations to produce biobutanol from lignocellulosic feedstocks via ABE fermentation.

3.2.2.1. Batch and fed-batch modes

The batch configuration, in which the entire substrate is used at once, is the easiest way to operate a fermentation process, demands minimum control, and reduces the contamination risk by other microorganisms. Therefore, this fermentation mode has been used for laboratory studies and industrial processes in Europe. However, this configuration is characterized by low productivities due to the long set-up periods and lag phases (Vees et al., 2020). Table 3.1 introduces a comparison of batch ABE fermentations using pure glucose or xylose with *C. acetobutylicum*, the species employed in this PhD thesis. In this table the butanol and ABE production, productivity and yield are showed.

The glucose is the main monosaccharide in lignocellulosic hydrolysates, hence most of the reported data has been carried out with this monosaccharide. For *C. acetobutylicum*, the employment of glucose as sole carbon source in batch mode usually results in productivities close to ~0.2 g L^{-1} h^{-1} while the butanol yield is normally in the range of 0.2 to 0.25 g g^{-1} (Table 3.1). The ABE fermentation with natural acidification resulted in different butanol productions depending mainly on the buffer formulation employed. Raganati et al. (2015) obtained up to 13.2 g L^{-1} of butanol and full glucose uptake (60 g L^{-1}) by buffering the media with 5 g L^{-1} of calcium carbonate. Yang et al. (2013) observed an acid crash with a butanol (ABE) production of 5.7 (~8.0) with less than 50% of glucose consumption employing a combination of ammonium acetate buffer and phosphates species (KH_2PO_4 and K_2HPO_4). This led to low butanol (ABE) productivities of ~0.08 (~0.11) g L^{-1} h^{-1}. To avoid acid crash, several strategies has been carried out. Yang et al. (2013) found that NaOH neutralization at the exponential growth phase promoted the full glucose consumption and almost doubled the butanol concentration and productivity up to 11.9 g L^{-1} and ~0.17 g L^{-1} h^{-1}, respectively. Luo et al. (2019) reported a butanol (ABE) production of 9.4 (13.7) g L^{-1} with glucose exhaustion in 31 h due to the coupling of an online pH control at 5.0 during acidogenesis. While, Li et al. (2011) observed a butanol production of ~11.0 g L^{-1} with a productivity of 0.37 g L^{-1} h^{-1},

one of the highest in literature for this kind of set-up, when the pH was controlled at a set-point of 4.5. These results clearly remark the importance of performing pH control strategies to avoid the excessive accumulation of the undissociated acid form with *C. acetobutylicum*.

Table 3.1. Comparison of batch ABE fermentation with *C. acetobutylicum* for butanol production using pure glucose or xylose as carbon source.

Chemical pH control	Butanol/ABE production (g L^{-1})	Butanol/ABE productivity (g L^{-1} h^{-1})	Butanol/ABE yield (g g^{-1})	References
Glucose				
No	13.2/19.2	0.26[2]/0.38[2]	0.24/0.35	Raganati et al. (2015)
No	5.7/8.0[1]	0.08[2]/0.11[2]	0.25[1]/0.35[1]	Yang et al. (2013)
Punctual	11.9/16.5[1]	0.17[2]/0.23[2]	0.24[1]/0.33[1]	
pH = 5.0	9.4/13.7	0.20[2]/0.28[2]	0.23[1]/0.26[1]	Luo et al. (2019)
pH = 4.5	11.0[1]/14.5[1]	0.37/0.52	0.24/0.35	Li et al. (2011)
Xylose				
No	7.8/10.8	0.05[2]/0.08[2]	0.19/0.26	Raganati et al. (2015)
pH = 5.1	7.0[1]/9.4	0.10[2]/0.13[2]	0.25[1]/0.34	Kudahettige-Nilsson et al. (2015)
pH = 5.0	8.0[1]/12.0[1]	0.08[2]/0.13[2]	0.16[1]/0.24[1]	Hu et al. (2011)

[1]Estimated from published data; [2]Calculated at the end of fermentation

Regarding the use of sole xylose, the secondary monosaccharide in lignocellulosic hydrolysates, the butanol productions are close to the inhibitory value with concentrations ranging from 7 to 8 g L^{-1} (Table 3.1). Raganati et al. (2015) reported an usage of 74% of xylose with a butanol production of 7.8 g L^{-1} using 60 g L^{-1} of xylose by buffering with 5 g L^{-1} of calcium carbonate. Meanwhile, Kudahettige-Nilsson et al. (2015) obtained a production of ~7 g L^{-1} of butanol with a 95% of total xylose utilization from 30 g L^{-1} at a pH set-point of 5.1. Other researchers found that using a pH set-point of 5.0 led to a nearly xylose depletion with a butanol production of ~8 g L^{-1} (Hu et al., 2011). In general, there is an agreement on literature that lower butanol productivities are obtained with xylose rather than glucose (0.05-0.10 g L^{-1} h^{-1}, Table 3.1). This can be attributed not only to the lower butanol production but also due to longer fermentation times required (normally >72 h) for the xylose metabolism. Thus, more studies are needed to better exploit the glucose and xylose with *C. acetobutylicum.*

The use of lignocellulosic hydrolysates in ABE fermentation implies previous steps of pretreatment and enzymatic hydrolysis that influences the outcome of the fermentation. The utilization of rice straw, the lignocellulosic feedstock used in this PhD

thesis, for ABE fermentation is scattered among microbial strains, pretreatments and enzymatic hydrolysis conditions employed (Table 3.2). Despite the disparity on the conditions used to produce butanol from rice straw hydrolysates the butanol yields are ~0.2 g g^{-1} (Table 3.2). These butanol yield values are similar to the data obtained with pure glucose or xylose as reported in Table 3.1. Moreover, the biobutanol productivities obtained with the rice straw hydrolysates are ~0.1 g L^{-1} h^{-1}, which coincide to the reported values with pure xylose as carbon source (Table 3.1). Moradi et al. (2013) compared the alkali and acid pretreatments with rice straw to produce biobutanol employing *C. acetobutylicum* NRRL B-591. These researchers obtained butanol productions of ~2.0 g L^{-1} in both pretreatments. Amiri et al. (2014) obtained 7.1 g L^{-1} of butanol with fully uptake of monosaccharides with *C. acetobutylicum* NRRL B-591 from a hydrolysate of organosolv pretreated rice straw. Researchers from the same research group managed to increment the butanol yield up to 0.31 g g^{-1} by changing the pretreatment and hydrolysis conditions with rice straw (Amiri et al., 2014; Moradi et al., 2013). Additionally, Wu et al. (2020) obtained a butanol production of 4.2 g L^{-1} from a monosaccharide consumption of 35 g L^{-1} using *C. beijerinckii* F-6. Furthermore, Valles et al. (2021b) used *C. beijerinckii* DSM 6422 with alkali pretreated rice straw to produce biobutanol. These authors obtained a butanol production of 10.1 g L^{-1} with full monosaccharide consumption and a maximum butanol (ABE) productivity of 0.19 (0.30) g L^{-1} h^{-1}, the highest value reported in Table 3.2. Moreover, the hydrolysate from rice straw pretreated with the protic ionic liquid of 2-hydroxyethylammonium acetate ([MEOA][OAc]) have been successfully fermented with *C. beijerinckii* DSM 6422. In these experiments, the use of fresh and recycled [MEOA][OAc] up to five times resulted in similar butanol productions of 7.3 g L^{-1} proving the reuse of the [MEOA][OAc] (Poy et al., 2022). The conversion of rice straw to butanol is also shown ranging from ~40 to ~80 g kg^{-1} of raw rice straw. The wider variability on the conversion of rice straw to butanol is due to the different severity of the pretreatments employed. In this sense, Chi et al. (2019) obtained the highest rice conversion to butanol with a value of 96 g kg^{-1} due to a solid recovery of64.7% in the pretreatment step despite getting a butanol production of 6.2 g L^{-1}. The results herein discussed demonstrate the feasibility of biobutanol production from rice straw hydrolysates.

Table 3.2. Comparison of batch ABE fermentation for butanol production using rice straw hydrolysates.

Microorganism	Pretreatment	Hydrolysis solid loading	Butanol/ABE production (g L^{-1})	Butanol/ABE Productivity (g L^{-1} h^{-1})	Butanol/ABE yield (g g^{-1})	Butanol/ABE yield (g kg^{-1})	References
C. acetobutylicum NRRL B-591	Alkali with NaOH	2% (wt/v)	1.4/2.0	0.02[1]/0.04[1]	0.20[2]/0.28[2]	45.2/64.1	Moradi et al. (2013)
	Acid with H_3PO_4	2% (wt/v)	2.0/2.9	0.02[1]/0.02[1]	0.20[2]/0.27[2]	44.2/63.0	
C. acetobutylicum NRRL B-591	Organosolv with ethanol + 1% H_2SO_4	8% (wt/wt)	7.1/10.5	0.10[1]/0.20[3]	0.31[2]/0.46[2]	80.3/123.9	Amiri et al. (2014)
C. beijerinckii F-6	Alkali with cold Urea/NaOH	3.8% (wt/v)	4.2/5.7[2]	0.15[3]/0.08[1]	0.13/0.18[2]		Wu et al. (2020)
C. beijerinckii NCIMB 8052	Alkali with NaOH	12% (wt/v)	6.2/9.0[2]	0.09[1]/0.13[1]	0.24/0.35[2]	96.0/139.4[2]	Chi et al. (2019)
C. beijerinckii DSM 6422	Alkali with NaOH	8% (wt/v)	10.1/16.7	0.19[3]/0.30[3]	0.24/0.39	77.6/126.7[2]	Valles et al. (2021b)
C. beijerinckii DSM 6422	Ionic liquid: [MEOA][OAc]	8% (wt/v)	7.3/12.0	0.10[1]/0.17[1]	0.21[2]/0.34[2]	81.7/134.3[2]	Poy et al. (2022)

[1]Calculated at the end of fermentation; [2]Estimated from published data; [3]Maximum productivity

Although fed-batch was not explored in this PhD thesis, it is an alternative for biobutanol production over the batch mode. The fed-batch mode is gaining importance due to it is able to increment the total quantity of feedstock metabolized. The fed-batch mode is carried out by the sequential addition of new substrate charges, so the microorganisms concomitantly consume the new carbon source. This stepped introduction of the carbon source allows to increment the total substrate metabolized avoiding substrate inhibition and/or the CCR phenomenon (Birgen et al., 2019; Ibrahim et al., 2018). Nevertheless, the fed-batch mode has similar downtimes as the batch mode, thus leading to comparable total productivities (Vees et al., 2020). Moreover, the use of this kind of operation with the ABE fermentation would require ISPR techniques to avoid butanol inhibition and to allow the conversion of the new substrate (Staggs and Nielsen, 2015). For instance, Lu et al. (2016) performed a fed-batch configuration coupled with a gas stripping ISPR resulting in a glucose consumption of 310 g L^{-1} and a solvent productivity and yield of 0.69 g L^{-1} h^{-1} and 0.48 g g^{-1} for *C. acetobutylicum* ATCC 824. Moreover, Valles et al. (2021a) developed a fed-batch simultaneous saccharification and fermentation with alkali pretreated rice straw coupled to a gas stripping. This configuration was able to double the butanol productivity (0.344 g L^{-1} h^{-1}) compared to the batch fermentation with *C. beijerinckii* DSM 6422 (Valles et al., 2021a).

3.2.2.2. Continuous mode

The continuous configuration is defined by the constant inflow and outflow of culture media. The main advantage of the continuous mode fermentation is the increase on the solvent productivities achievable in comparison with the batch mode. The efficiency of these systems is also increased by the reduced lag phase and overall downtime while reducing the product inhibition. Moreover, this fermentation mode provides steady effluents which facilitates the downstream process. Nevertheless, this kind of set-up requires more process control over batch modes, such as, tight anaerobic conditions or avoidance of microbial contamination (Ibrahim et al., 2018; Vees et al., 2020). Another drawback of this configuration, especially on ABE fermentation, is the strain degeneration. In this case, the solventogenic *Clostridium* spp. could lose the ability to produce solvents due to the long-term operation (Ennis and Maddox, 1989; Liu et al., 2019a).

The simplest continuous set-up is the continuous stirred tank reactor (CSTR) in which the cells grow in suspension. However, this kind of configuration is limited by the maximum growth rate of the microorganisms which restrains the maximum dilution rate employed. The combination of the restriction of the dilution rate along with the low cell

concentration in the fermentation broth hinders the volumetric solvent productivity in CSTRs. Moreover, the by-phasic nature of the ABE fermentation also interferes the performance of the continuous operation in suspended growth (Ibrahim et al., 2018; Vees et al., 2020). The CSTR configuration can be modified to overcome its limitations. Multi-stage systems can be developed that connect several reactors in series. The most common multi-stage system with solventogenic *Clostridium* spp. is the separation of the acidogenic and solventogenic phases by setting different pHs for each metabolic stage of the fermentation (Godin and Engasser, 1990; Raganati et al., 2016). In addition, the strategies to retain or increment the available biomass allow to uncouple the microbial growth over the inflow liquid surpassing one of the limitations of the CSTR. The techniques aimed to augment the biomass in ABE fermentation may raise the product titer and productivity and the consumption of substrate (Survase et al., 2013). The accumulation of the biomass in the reactors may be accomplished by cell retention techniques (Survase et al., 2019), cell immobilization (Dolejš et al., 2014; Survase et al., 2013) or biofilm reactors (Raganati et al., 2022).

The augmentation of biomass via cell immobilization or biofilm reactors creates a protective environment to the microorganisms over the sheering forces, the possible inhibitors in the fermentation broth as the hydrolysate toxic compounds or the alcohols themselves compared to the suspended growth (Liu et al., 2013; Vees et al., 2020). The drawbacks of these configurations are normally the occurrence of microenvironments which can limit the internal diffusion and the overgrowth of microbial biomass which can lead to fouling (Vees et al., 2020; Zhang et al., 2021). A wide range of immobilization supports can be employed to increment the available biomass. The matrixes deployed can be inert rings (Raganati et al., 2022, 2016); activated carbon (Jin et al., 2020; Liu et al., 2019b); bricks (Liu et al., 2019b; Qureshi et al., 2000); natural fibres (cotton meshes, linen or the lignocellulosic materials themselves) (Liu et al., 2019a; Survase et al., 2013, 2012); calcium alginate (Ye et al., 2018); or porous polyvinyl alcohol (S.-M. Lee et al., 2008), among others.

The continuous butanol production with *C. acetobutylicum* and glucose as carbon source has been reported in a range of continuous configurations. Li et al. (2011) doubled the butanol (ABE) productivity from a value of 0.37 (0.52) g L^{-1} h^{-1} in batch mode to a value of 0.81 (0.92) g L^{-1} h^{-1} in a CSTR operated at a dilution rate of 0.12 h^{-1} when both batch and CSTR reactors were pH-controlled at 4.5. Moreover, Dolejš et al. (2014) increased the butanol (ABE) productivity from 0.10 (0.14) to 0.33 (0.50) g L^{-1} h^{-1} by changing the batch set-up to a free-cell continuous reactor (dilution rate of 0.11 h^{-1}) with

20 g L^{-1} of glucose and *C. acetobutylicum* DSM 1731. The multi-stage operation in ABE fermentation has been also explored. Bahl et al. (1982) operated a two-stage phosphate limited chemostat using *C. acetobutylicum* DSM 1731. A pH value of 4.3 was found optimal in both stages resulting in a butanol production of ~12.6 (~18.3) g L^{-1} in the second reactor and a butanol (ABE) productivity of ~0.30 (~0.44) g L^{-1} h^{-1} with an overall dilution rate of 0.025 h^{-1}. Whereas, Lai and Traxler (1994) performed a two-stage continuous fermentation with *C. acetobutylicum* ATCC 824. The first reactor was operated with a limitation of nitrogen and an excess of glucose at pH = 6.0 to obtain a rich acid effluent. This effluent rich in acids was supplemented with nitrogen and glucose while fed to the second reactor with no pH control dropping to a value of 4.5. Under these conditions, the butanol (ABE) productivity from an overall dilution rate of 0.033 h^{-1} was 0.2 (0.3) g L^{-1} h^{-1}. As reported, the fermentations with free-cells usually result in butanol and solvent productivities <1 g L^{-1} h^{-1}. Thus, other alternatives may be required to further increment the volumetric productivities.

The use of cell retention techniques on continuous ABE fermentation with *C. acetobutylicum* has been reported to increase solvent productivities. Chang et al. (2014) evaluated the influence of sweet sorghum bagasse as immobilization support with *C. acetobutylicum* ABE 1201. These researchers increased the butanol (ABE) productivity from ~0.22 (~0.37) g L^{-1} h^{-1} in batch mode to ~0.72 (~1.32) g L^{-1} h^{-1} in a continuous reactor (dilution rate of 0.08 h^{-1}) by employing the same carrier in both reactors. Moreover, Bankar et al. (2012) developed a continuous two-stage continuous ABE formation with sugarcane bagasse as carrier and a liquid-liquid ISPR technique between stages. The maximum solvent productivity was 2.5 g L^{-1} h^{-1} using an overall dilution rate of 0.2 h^{-1} (first reactor: 0.6 h^{-1} and second reactor: 0.3 h^{-1}). Furthermore, Survase et al. (2012) developed an immobilized column reactor with wood pulp using *C. acetobutylicum* DSM 792 and glucose as carbon source. These researchers evaluated a range of dilution rates from 0.22 to 2.4 h^{-1}, in which the dilution rate of 1.9 h^{-1} resulted in the maximum solvent productivity of 13.7 g L^{-1} h^{-1}. Raganati et al. (2022) performed a multi-stage configuration consisting on four packed bed biofilm reactors in which the reactor was set at pH 5.5 to promote acidogenesis and the rest at 4.7 to promote solventogenesis. These researchers observed that an overall dilution rate of 0.90 h^{-1} achieved a maximum butanol productivity of ~13 g L^{-1} h^{-1} with 100 g L^{-1} of glucose as carbon source. These researchers almost doubled the butanol productivity to 22 g L^{-1} h^{-1} when an adsorption column was coupled as ISPR technique between the third and fourth reactor. Nevertheless, the glucose concentration was much higher than the usual monosaccharide composition in lignocellulosic hydrolysates. The results herein shown

that biobutanol productivity can be incremented when operating under continuous mode with *C. acetobutylicum*.

The performance of continuous ABE fermentation with real lignocellulosic feedstocks remains scarce. Table 3.3 summarizes some examples relative to the development of continuous operation with solventogenic *Clostridium* spp. and lignocellulosic feedstocks. For wheat straw, Van Hecke et al. (2017) employed a self-developed clostridial strain (GBL-B) to produce biobutanol. These researchers obtained a butanol (ABE) productivity of 0.18 (0.25) g L^{-1} h^{-1} in a CSTR coupled to a pervaporation unit operated at a pH set-point of 4.8 and a dilution rate of 0.0137 h^{-1}. For de-oiled rice bran, Al-Shorgani et al. (2019) reported a butanol (ABE) productivity of 0.14 (0.25) g L^{-1} h^{-1} using a CSTR with free cells of *Clostridium acetobutylicum* YM1. For corn stover, Ni et al. (2013) obtained a butanol (ABE) productivity up to 0.29 (0.43) g L^{-1} h^{-1} with an overall dilution rate of 0.0375 h^{-1} in a four-stage continuous set-up under suspended cells of *Clostridium saccharobutylicum* DSM 13864. For pine chips, Survase et al. (2019) divided the hydrolysates into a hexose or pentose rich liquids. Both hydrolysates were fermented into two different CSTR with an online chemical pH control (set-point of 4.8) coupled with cell recycling module and the modified strain *AvapClo*™. This mode of operation let to obtain a solvent productivity of 12.1 g L^{-1} h^{-1} at a dilution rate of 0.77 h^{-1} with the hexoses rich hydrolysate. Meanwhile, using the pentoses rich hydrolysate was obtained a solvent productivity of 6.1 g L^{-1} h^{-1} at a dilution rate of 0.66 h^{-1}. For spruce chips, Survase et al. (2011) reported a maximum butanol (ABE) productivity of ~2.9 (4.9) g L^{-1} h^{-1} at a dilution rate of 0.64 h^{-1} with a column reactor with wood pulp as carrier and *C. acetobutylicum* DSM 792. The results herein depicted demonstrate the feasibility of continuous butanol production with lignocellulosic hydrolysates. However, the use of rice straw as lignocellulosic feedstock has not been employed in continuous operations yet.

Table 3.3. Continuous configurations with hydrolysates from several lignocellulosic feedstocks for solvent production by solventogenic *Clostridium* spp.

Microorganism	Lignocellulosic feedstock	Configuration	Dilution rate (h^{-1})	Butanol/ABE production (g L^{-1})	Butanol/ABE productivity (g L^{-1} h^{-1})	References
GBL- B[1]	Wheat straw	CSTR[2]	0.0137	12.9/18.3	0.18/0.25	Van Hecke et al. (2017)
C. acetobutylicum YM1	De-oiled rice bran	CSTR	0.0200	6.9/12.4	0.14/0.25	Al-Shorgani et al. (2019)
C. saccharobutylicum DSM 13864	Corn stover	Four-stage CSTR	0.0375[3]	7.8/11.4	0.29/0.43	Ni et al. (2013)
Modified strain *AvapClo*™ [4]	Pine wood[5]	CSTR with cell recycling	0.7700	8.7[6]/15.6	6.70[6]/12.10	Survase et al. (2019)
	Pine wood[7]		0.6600	6.3[6]/11.2	4.20[6]/6.10	
C. acetobutylicum DSM 792	Spruce chips	Column reactor with wood pulp as carrier	0.6400	4.5[6]/7.6[6]	2.90[6]/4.90	Survase et al. (2011)

[1] Clostridial strain from Green Biologics; [2] Coupled with POMS pervaporation membrane; [3] Overall dilution rate; [4] *C. acetobutylicum* ATCC 824 with *ADH* gene; [5] Hydrolysate rich in hexoses; [6] Estimated from published data; [7] Hydrolysate rich in pentoses

3.2.3. Co-cultures systems for butanol production using lignocellulosic feedstocks

The performance of co-cultures systems is a strategy that may enhance the performance of microbial fermentations. In this section, the development of co-cultures systems with *Clostridium* spp. and lignocellulosic biomasses is explained as a way to increase solvent production. A co-culture can be described as the incubation of two or more defined microorganism under aseptic conditions (Bader et al., 2010). The interactions among microorganisms and their surrounding can shift depending on the external conditions creating different behavioural networks to produce different chemical compounds as observed in Figure 3.2. In this figure is depicted the interaction of mono-cultures, co-cultures and mixed cultures. A mixed culture is defined as the cultivation of different undefined microorganisms (Bader et al., 2010). The performance of artificial co-cultures creates new opportunities as the combination of microorganisms can degrade more complex substrates. Indeed, the chemical output spectrum of the fermentation can be widened due to the presence of more metabolic pathways. Another potential advantage would be that co-culture systems may increment the production rates. Moreover, the microbial co-cultures may endure environmental disturbances, thus increasing the system robustness, as opposed to the pure cultures in which oscillations on the culture conditions may hinder the fermentation performance as microorganisms have very specific optimal conditions (Hasibar et al., 2020).

The consolidated bioprocessing is the most important co-culture approach with lignocellulosic feedstocks. This configuration integrates a hydrolytic microorganism capable of breaking a macromolecular structure, like the lignocellulosic biomass, and one microorganism that will feed from the released monosaccharides in a single vessel. Most of the lignocellulolytic bacteria are enclosed in the phylum Bacillota (former Firmicutes) that includes the *Clostridium* spp. (Singhania et al., 2022). In this regard, a consolidated bioprocessing using alkali-pretreated rice straw with *Clostridium thermocellum* (current name *Acetivibrio thermocellus*) and *C. saccharoperbutylacetonicum* was able to produce 5.5 g L^{-1} of butanol (Kiyoshi et al., 2015). Moreover, the consolidated bioprocessing of corn cob with *Thermoanaerobacterium thermosaccharolyticum* and *C. acetobutylicum* NJ4 resulted in a butanol production of 7.6 g L^{-1} employing 180 g L^{-1} of the lignocellulosic waste (Jiang et al., 2020).

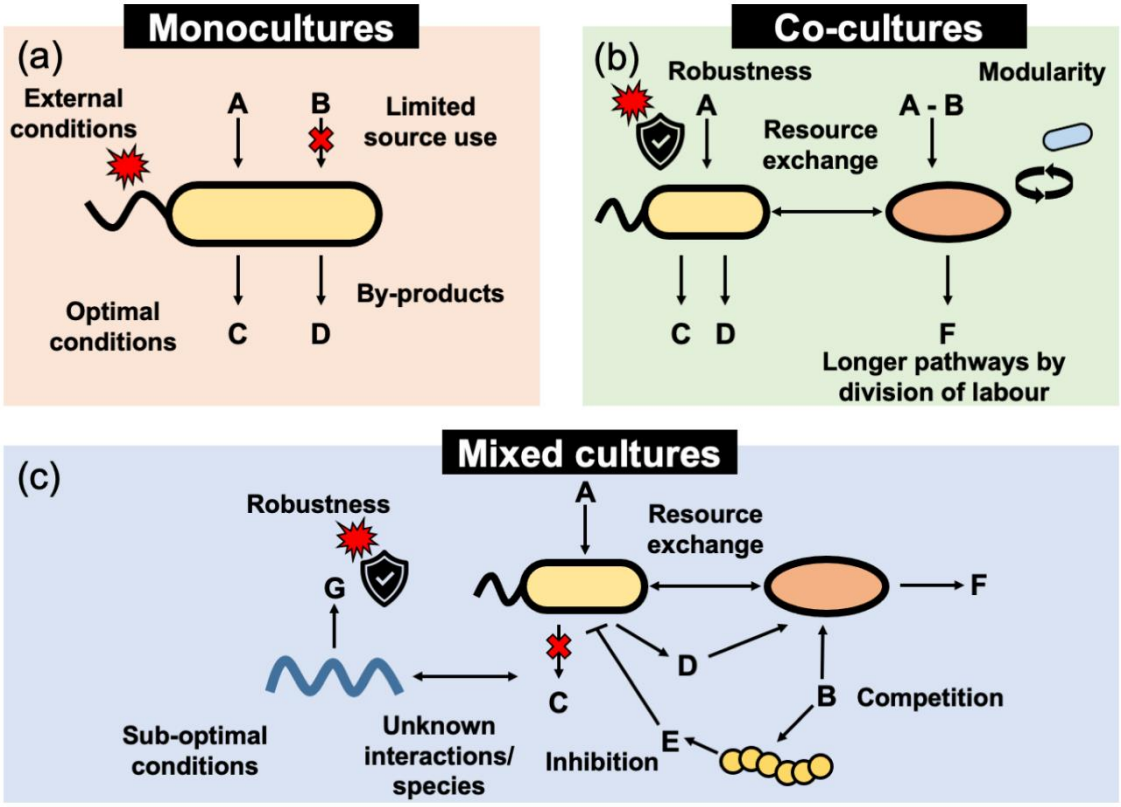

Figure 3.2. Conceptual scheme on the characteristics and interactions for microbial populations. (a) Monocultures; (b) Synthetic co-cultures and (c) Mixed cultures. Adapted from Diender et al. (2021).

Another approach for using co-cultures with *Clostridium* spp. would focus on looking for other specific benefits due to the metabolic capacities of the class Clostridia. In that context, the culture with aerobic or facultative anaerobic bacteria can neutralize oxygen and create anaerobic conditions favouring the microorganism growth and reducing the necessity of inert gases or reducing agents. For instance, the performance of a co-culture with *Bacillus subtilis* and *C. acetobutylicum* using agave hydrolysate incremented the butanol production from 6.1 to 8.2 g L^{-1} over the monoculture. This increment was related to the oxygen depletion and the hydrolysate detoxification ability of the *B. subtilis* as may degrade inhibitors coming from the biomass pretreatment (Oliva-Rodríguez et al., 2019).

The performance of the solventogenic *Clostridium* spp. can also be improved by the synergetic effect of other microorganisms due to their excretion of growth factors or other compounds. In this sense, the culture of *C. acetobutylicum* with exogenous additions of a mixture of valine:arginine (20:80, 1 g L^{-1}) or tryptophan:phenylalanine (80:20, 1 g L^{-1}) have been found to increment the butanol production from 6.9 to more

than 10 g L^{-1} when added in the early stages of the fermentation (Nimbalkar et al., 2019). Following this, the yeast *S. cerevisiae* is capable of excreting amino acids which can be posteriorly uptaken by the *Clostridium* spp. increasing the transport of monosaccharides and the solvent tolerance (Luo et al., 2015; Wu et al., 2019). The *S. cerevisiae* is unable to consume pentoses, which hinders its performance from lignocellulosic hydrolysates to produce bioethanol via the alcoholic fermentation (Figure 3.3). Nevertheless, *S. cerevisiae* may present tolerance to the most common inhibitors found in lignocellulosic hydrolysates which raises its interest to carry out co-culture fermentations with this kind of carbon sources. For instance, *S. cerevisiae* Ethanol Red can metabolize some of the typical inhibitors produced during the pretreatment of lignocellulosic biomasses, such as, 5-HMF, furfural and vanillin when fermented in either aerobic or anaerobic conditions (Kłosowski and Mikulski, 2021).

The strategy of combining solventogenic *Clostridium* spp. and *S. cerevisiae* has been mainly evaluated with the use of feedstocks rich in glucose as starchy substrates. For instance, Luo et al. (2017) raised the butanol (ABE) production from 7.6 (12.7) to 9.5 (18.6) g L^{-1} by employing 20 g L^{-1} of initial glucose and 15% of corn flour with *C. acetobutylicum* ATCC 824 and *S. cerevisiae*. Furthermore, Qi et al. (2018) substantially incremented the ABE production from 17.7 to 42.56 g L^{-1} by the utilization of 150 g L^{-1} of cassava flour with *C. acetobutylicum* CH02 and *S. cerevisiae*. The remarkable higher solvent production was mainly due to the ethanol production increment (1.5 to 27.2 g L^{-1}) in the same fermentation pot. Hence, a better usage of the available carbon source was obtained by the co-culture fermentation of these microorganisms over the *C. acetobutylicum* monoculture. Nevertheless, the development of co-culture strategies such as the combination of *Clostridium* spp. with *S. cerevisiae* is still scarce and under research in order to improve the exploitation of lignocellulosic feedstocks. In this PhD thesis, a co-culture system of *C. acetobutylicum* and *S. cerevisiae* was carried out with rice straw hydrolysate for the exploitation of the lignocellulosic waste.

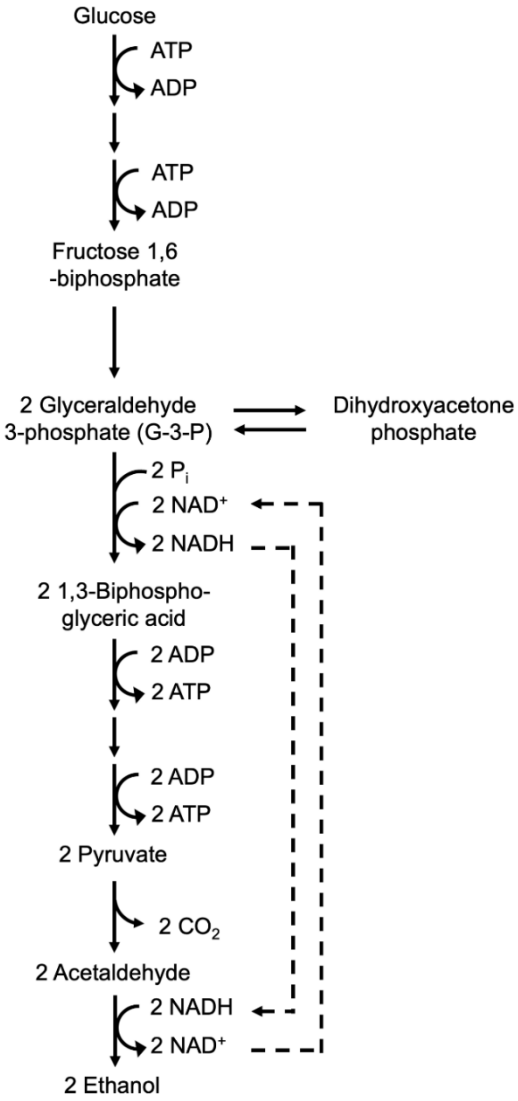

Figure 3.3. Scheme of the metabolic pathway for the alcoholic fermentation by *S. cerevisiae.* Adapted from Acorsi et al. (2022).

3.3. HBE fermentation

Other solventogenic *Clostridium* spp., which are enclosed in the acetogens group of microorganisms, can convert CO or syngas/waste gas streams into biomass via the Wood-Ljungdahl pathway (WLP) (Figure 3.4, purple box). This pathway has two different branches depending on the carbon gas species. The “Eastern” or methyl branch where formate is produced by the reduction of CO_2 using H_2 as electron donor. Meanwhile, the “Eastern” or carbonyl branch in which the CO is directly incorporated. Both routes will converge in the synthesis of acetyl-CoA that will be used to generate the end products (Figure 3.4). Among this acetogenic cluster of bacteria, it can be found *C.*

carboxidivorans, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium drakei or *C. autoethanogenum.*

Among these bacteria, *C. carboxidivorans* was originally isolated from an agricultural settling lagoon in 2005 (Liou et al., 2005). This microorganism rises of interest due to its capability of performing the HBE fermentation to produce bioalcohols. The HBE fermentation produces short and middle carbon chain products (acids/solvents) such as, acetic acid/ethanol (two carbons, C2), butyric acid/butanol (C4) and hexanoic acid/hexanol (C6) following the characteristic biphasic fermentation of solventogenic *Clostridium* spp. (Figure 3.4) (Fernández-Naveira et al., 2017a). *C. carboxidivorans* is capable of growing on either monosaccharides or CO-rich gases (Fernández-Naveira et al., 2017b, 2017c). Nevertheless, research is currently focused on employing gas streams rich in CO for the production of bioalcohols.

Identically to the ABE fermentation, the production of HBE solvents from *C. carboxidivorans* is strongly influenced by several factors as the pH or the carbon source. For instance, *C. carboxidivorans* has optimum growth range between 5.0 to 7.0 for developing the HBE fermentation (Fernández-Naveira et al., 2017a). Its biphasic fermentation can be modulated by pH control. A constant pH of 5.75 has been reported to intensify the acid concentration compared to the uncontrolled batch reactors. Additionally, the maintenance of the set-point at 5.75 during the whole experiment increased the total solvent production due to the higher acid production and posterior reassimilation compared to natural acidification to 4.75 after initial 24 h of pH control at 5.75 (Fernández-Naveira et al., 2017c).

The use of CO-rich gases also involves several drawbacks such as the low solubility of the gases in the fermentation media or the CO toxicity on hydrogenases (Fernández-Naveira et al., 2017a). In this regard, Lanzillo et al. (2020) observed that the partial pressure of CO altered the kinetics of *C. carboxidivorans* in batch bottles. The growth rate of this microorganism was inhibited by CO in the range of partial pressures from 0.5 to 2.5 atm. The optimum partial pressure of CO was 1.1 atm, equivalent to a concentration of dissolved CO of ~25 mg L^{-1}.

Additionally, *C. carboxidivorans* grown on pure CO presents a half-maximum inhibitory concentration of 14.5 g L^{-1} for butanol after 48 h with no growth at 20 g L^{-1} of butanol, while a half-maximum inhibitory concentration of ~35 g L^{-1} was estimated for ethanol (Fernández-Naveira et al., 2016a). Remarkably, *C. carboxidivorans* exhibits a better tolerance to butanol (20 g L^{-1}) rather than *C. acetobutylicum* (13-14 g L^{-1}).

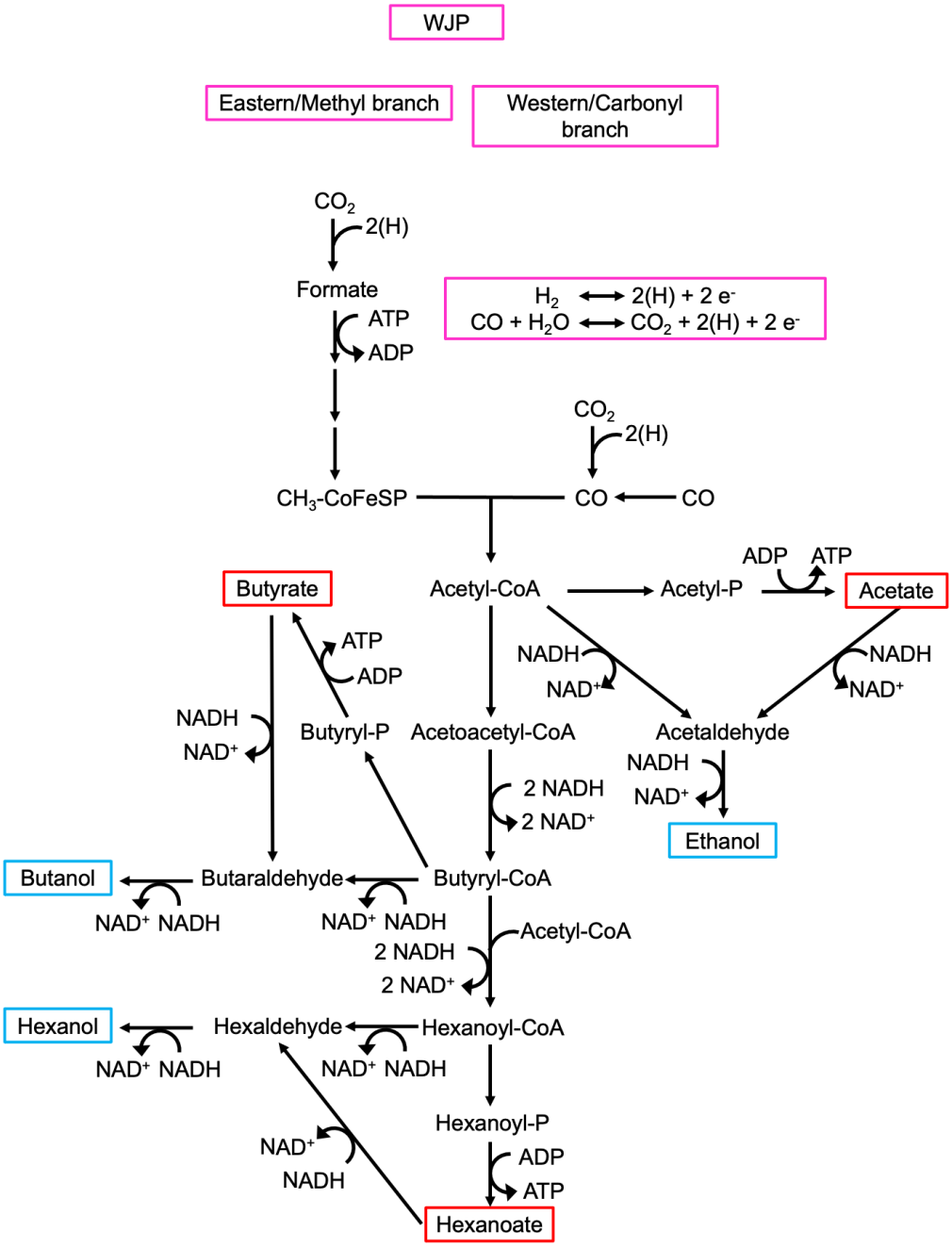

Figure 3.4. Scheme of the metabolism of the HBE fermentation by *C. carboxidivorans*. WLP (purple box). Red box: products of acidogenesis. Blue box: products of solventogenesis. Adapted from Fernández-Naveira et al. (2017a) and Vees et al. (2020).

3.3.1. Continuous mode

In this section, it is described the state of the art for the continuous configuration to produce HBE bioalcohols from syngas. The main continuous configuration employed on syngas fermentations is the CSTR due to its advantageous mixing conditions leading into good mass transfer between the phases required for gas fermentations. Moreover,

this set-up shows a stable gas fermentation when working with acetogens as *C. carboxidivorans*. Indeed, no solvent degeneration in acetogenic bacteria has been reported working with one-stage CSTRs (Vees et al., 2020). Hence, differences in biological behaviour may be observed when working with solventogenic and acetogenic *Clostridium* spp.

Table 3.4. Continuous configurations for production of chemicals of interest (acids and HBE solvents) with *C. carboxidivorans.*

Carbon source	Set-up	Overall dilution rate (h^{-1})	Product	Titer ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ day^{-1}$)	Reference
$CO:CO_2$ (60:40 vol%)	Two-stage CSTR	0.048[1]	Ethanol Butanol Hexanol	6.1 0.7 0.1	7.00 0.84 0.08	Doll et al. (2018)
$CO:CO_2:H_2:N_2$ (30:10:20:40 vol%)	Two-stage CSTR with cell recycling	0.017	Ethanol Butanol Hexanol	1.0[1] 0.5[1] 0.2[1]	0.41[1] 0.20[1] 0.08[1]	Abubackar et al. (2018)
Glucose (10 $g\ L^{-1}$) + CO (20 vol%)	CSTR	0.05	Ethanol Butanol Hexanol	5.7 2.6 0.7	~6.8[1] ~3.1[1] ~0.8[1]	Vees et al. (2022)

[1]Estimated from published data

The continuous gas and liquid fermentation by *C. carboxidivorans* has been proven feasible as shown in Table 3.4. In this sense, Doll et al. (2018) successfully performed a continuous cascade two CSTRs and two pH levels of 6.0 (first reactor, dilution rate of 0.12 h^{-1}) and 5.0 (second reactor, dilution rate of 0.08 h^{-1}) with an overall dilution rate of ~0.048 h^{-1}. Under these conditions, an ethanol, butanol and hexanol productivity of 7.00, 0.84 and 0.08 g L^{-1} day^{-1} were accomplished, respectively. The high solvent productivity, especially ethanol but also hexanol, under these operational parameters is of importance to obtain a profitable way to valorise syngas mixtures. Abubackar et al. (2018) used a two-stage CSTR (pH set-point of 6.0 and 5.0 for first and second reactor) coupled with a biomass recirculating module in the solventogenic reactor. In this case, a hexanol productivity of ~0.08 g L^{-1} day^{-1} was obtained with a dilution rate of 0.017 h^{-1}. Furthermore, Vees et al. (2022) evaluated the mixotrophic conversion of glucose and CO in a one-stage CSTR with a pH set-point of 6.0 and a dilution rate of 0.05 h^{-1}. These researchers incremented the hexanol production from 0.5 to 0.7 g L^{-1} when feeding a mixotrophic input (10 g L^{-1} of glucose and 20% CO) over the heterotrophic input (10 g L^{-1}) using *C. carboxidivorans*. The good performance in terms of solvent production would be related to the glucose availability in which the mixotrophic

also enhanced the ethanol and butanol production by 1.7 and 1.5 fold, respectively (Table 3.4). More research is required for the applicability of continuous syngas fermentations to produce HBE solvents.

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4. OBJECTIVES

The main objective of this PhD thesis is to investigate the biobutanol and biohexanol production by fermentation from solventogenic *Clostridium* spp. in order to explore new alternatives for the exploitation of lignocellulosic waste in the framework of Circular Economy. The general objective comprised the following partial objectives:

1) To obtain a batch model ABE fermentation for the exploitation of monomeric sugars contained in the lignocellulosic waste, such as, rice straw in order to produce biobutanol by *C. acetobutylicum* DSM 792. Main novelty aspects for the enhancement of the process are:

    - By establishing initial levels of glucose within ranges from lignocellulosic hydrolysates, the combined effect of the initial level of the monomeric sugar and the pH regulation strategy on butanol production will be investigated. Two pH regulation strategies will be evaluated 1) using an initial dose of calcium carbonate as a buffer and 2) implementing an online control of the minimum pH value.

    - From the results with glucose, the model will be extended to the fermentation of xylose, the secondary monosaccharide of rice straw. Carbon source formulations of pure xylose and monosaccharide mixtures of glucose and xylose mimicking rice straw hydrolysates will be used to establish the pH regulation strategy (buffer formulation with/without online pH control) that maximises butanol production.

2) To perform a proof of concept of a continuous fermentation with cell retention and online pH control as an alternative for increasing butanol productivity comparing to batch configuration. For this purpose, initially glucose as model substrate will be used during start-up, and afterwards, a mixture of glucose and xylose that mimicked rice straw hydrolysate will be employed.

3) To validate the batch model fermentation on rice straw hydrolysate by using buffered media with and without online pH control.

4) To evaluate the use of a co-culture batch process as a means to increase the exploitation of rice straw hydrolysate into ABE solvents. For that purpose, the best elapsed time between the inoculation of both

microorganisms (*C. acetobutylicum* DSM 792 and *Saccharomyces cerevisiae* ESY4) will be evaluated. The concept will be developed with synthetic media composed of monomeric sugars mimicking the composition of rice straw hydrolysate. The model will be validated with rice straw hydrolysate. Both pH regulation strategies developed on this thesis will be tested (buffered media with/without online control of minimum pH).

5) To investigate an alternative *Clostridium* fermentation route based on the previous conversion of rice straw to syngas for its exploitation into middle carbon chain bioalcohols. The research objective will be to assess the feasibility of a continuous fermentation configuration by using *C. carboxidivorans* DSM 15243 from carbon monoxide, main component of syngas. Specifically, the highest dilution rate that maximised hexanol production under pH-controlled processes will be determined.

5. GENERAL MATERIALS AND METHODS

In this chapter are described the main equipment, experimental procedures and analytical techniques used during the work of this PhD thesis.

5.1. Reagents

All the chemicals employed in this PhD thesis were obtained by regular suppliers and were of chemical or analytical grade depending on their use. The gasses employed during this work were of +99.9% purity.

5.2. Rice straw

The rice straw feedstock was obtained from farmers of the L'Albufera Natural Park in Valencia (Spain). The composition of the original rice straw in dry weight is shown in Table 5.1 (Poy et al., 2021). The rice straw was stored at room temperature up to use.

Table 5.1. Composition of rice straw in dry weight.

Component	% (w/w)
Glucan	35.8 ± 2.1
Xylan	14.8 ± 1.6
Arabinan	2.7 ± 0.4
Lignin	14.3 ± 0.3
Extractives	13.2 ± 2.1
Ashes	16.7 ± 0.1
Acetyl groups	1.0 ± 0.1

Prior to fermentation, both pretreatment and hydrolysis steps are required to liberate the monosaccharides. The pretreatment and the enzymatic hydrolysis protocols used in this PhD thesis were previously developed in our laboratory (Valles et al., 2021b, 2020). About the pretreatment step, it consisted in a size reduction followed by alkali pretreatment with NaOH. Rice straw was milled (Taurus Optima 1300, Taurus S.L., Spain) and sieved (ISO-3310.1, CISA, Spain) to a particle size range of 100 to 2000 µm. Alkaline pretreatment conditions were 0.75% (w/v) of NaOH and 5% (w/w) solid loading in an autoclave (134 ºC, 40 minutes, MED20, J.P. Selecta, Spain). The solid fraction was hereinafter washed and dried at 45 ºC prior storage at 4 ºC. The morphological change of the feedstock during the pretreatment can be seen in Figure 5.1 along with the further changes of the slurry (enzymatic hydrolysis, ABE fermentation) up to the final fermented product.

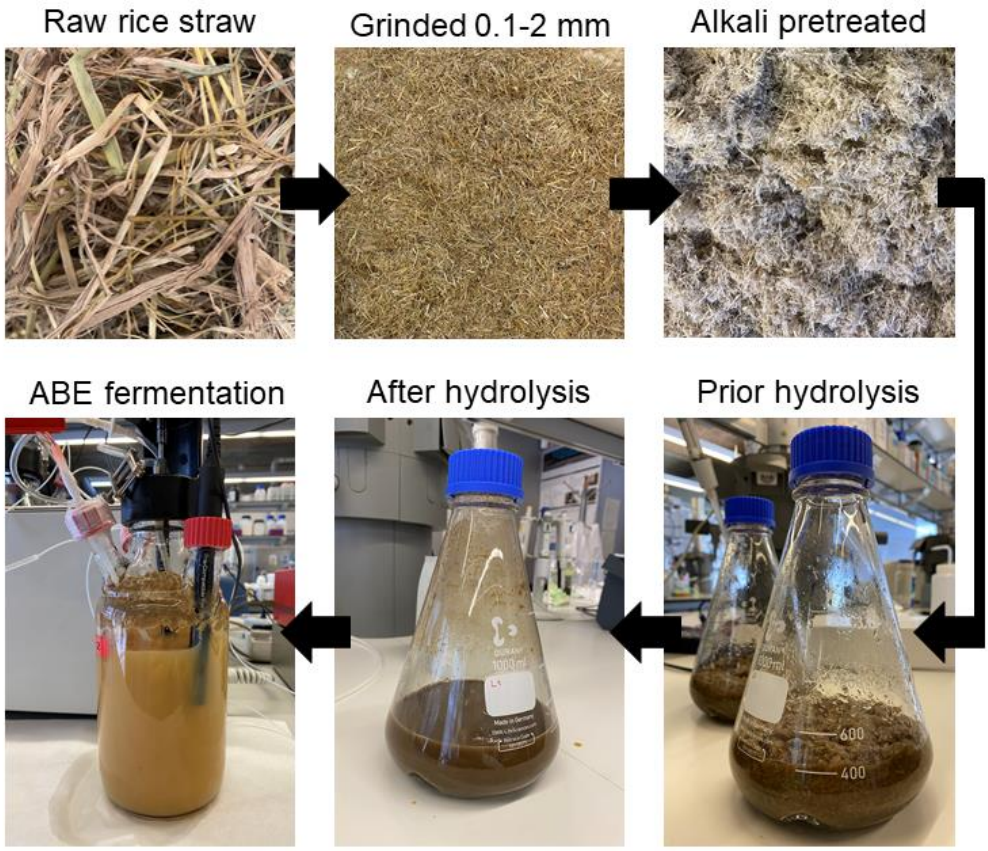

Figure 5.1. Morphological changes of the rice straw feedstock during the whole process from raw rice straw to the fermented product.

Enzymatic hydrolysis was carried out with the commercial enzyme blend Cellic CTec2 (Novozymes, Denmark) in an orbital shaker for 72 h (G25, New Brunswick scientific, USA) with the conditions summarized in Table 5.2. After hydrolysis, the liquid was filtered by 10-20 µm and stored at -4 ºC. The composition of the rice straw hydrolysate used in the experiments of this doctoral thesis is shown in Table 5.3. The quantification of the enzymatic activity of the commercial blend Cellic CTec2 was performed following the standard procedure of the National Renewable Energy Laboratory from USA (Adney and Baker, 1996).

Table 5.2. Enzymatic hydrolysis conditions for the pretreated rice straw with the commercial enzyme blend Cellic CTec2.

Parameter	Value
Solid loading	8% (w/v)
Enzyme loading	20 FPU/g-dw
Buffer	50 mM sodium acetate
pH	5.2
Temperature	50 ºC
Stirring	150 rpm

Table 5.3. Composition of the rice straw hydrolysate.

Compound	Concentration (g L^{-1})
Glucose	38.3 ± 1.2
Xylose	14.0 ± 0.4
Cellobiose	4.5 ± 0.2
Arabinose	1.6 ± 0.4
Acetic acid	4.3 ± 0.5
Phenolic compounds	0.15 ± 0.01
Levulinic acid	N/D
Furfural	N/D
5-HMF	N/D

N/D denotes no detected.

5.3. Microorganisms

The strain *C. acetobutylicum* DSM 792 was purchased lyophilized from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* - DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The lyophile was re-activated following the procedure provided by DSMZ. The active culture was later cryopreserved at -80 ºC in 39 g L^{-1} of Reinforced Clostridial Medium (RCM, VWR, Belgium) with glycerol (20% v/v, Fischer Scientific, USA).

The yeast *S. cerevisiae* EYS4 was kindly donated in agar plates from the collection of Emilia Matallana and Agustín Aranda (I2SysBio UV-CSIC, Burjassot, Spain). The selection was made by these researchers as a good candidate for fermentation at 37 ºC (Grijalva-Vallejos et al., 2021, 2020). The yeast was maintained on Yeast-Peptone-Dextrose (YPD) agar at 4 ºC. The agar plates were composed of yeast extract (10 g L^{-1}, Thermo scientific, USA); bacteriological peptone (20 g L^{-1}, PanReac AppliChem, Spain); dextrose (20 g L^{-1}, Thermo scientific, USA) and agar (15 g $L^{-1,}$ PanReac AppliChem, Spain).

Active culture of *C. carboxidivorans* DSM 15243 (strain designation P7) was purchased from DSMZ (Braunschweig, Germany). The active culture was cryopreserved at -80 ºC in 33 g L^{-1} Wilkins Chalgren Anaerobic Broth (WCAB, Sigma-Aldrich, Italy) with glycerol (20% v/v, Sigma-Aldrich, Italy).

5.4. Culture propagation

Serum bottles (50 mL) were used to propagate the inoculum of *C. acetobutylicum*. The bacterium was cultured in 40 mL of working volume with 19 g L^{-1} of RCM fortified with 10 g L^{-1} of glucose. Anaerobic conditions were accomplished by

insufflating N_2 (99.99%, Carburos Metálicos S.A., Spain) during several minutes. The serum bottles were sealed with an aluminium crimp cap (Fischer Scientific, Denmark) and a butyl/PTFE septum (Supelco, USA) and sterilized by autoclaving (121 ºC, 20 minutes, MED20, JPSelecta, Spain). Afterwards, the serum bottles were inoculated with 100 µL from a cryotube with the glycerol stock. Growth was carried out statically at 37 ºC (INB 200, Memmert, Germany).

Erlenmeyer flasks (250 mL) were used to prepare active cultures of *S. cerevisiae*. The yeast was cultured in 50 mL of YPD broth volume. The medium was sterilized by autoclaving (121 ºC, 20 minutes, MED20, JPSelecta, Spain). Flasks were inoculated with a 5% (v/v) of active microorganism. Growth was carried out at 37 ºC and 150 rpm (G25, New Brunswick scientific, USA).

Hungate tubes (15 mL) were used for the inoculum propagation of *C. carboxidivorans*. The microorganism was cultured in 12 mL of WCAB with 0.6 g L^{-1} of L-cysteine. The medium was heated up to 99 ºC for 10 minutes, after which anaerobic conditions were accomplished by flushing N_2 (99.9%) until cooling down and then the reducing agent, L-cysteine, was added. The tubes were sterilized by autoclave (121 ºC, 20 minutes, NC 40M, Nüve, Turkey) and inoculated with 0.5 mL from a cryotube. Growth was carried out at 35 ºC and 130 rpm (Shaker Incubator SKI 4, Argo Lab, Italy).

5.5. Fermentation configurations

During the development of this doctoral thesis several fermentation configurations were employed.

5.5.1. Batch fermenter

The main set-up employed in this PhD thesis was a fermenter operated in batch mode. It was used in the experiments described in Chapters 6, 7 and 8. Assays were carried out using 0.8 L of working volume in a 1.1 L total volume reactor. The experimental set-up is depicted in Figure 5.2. It included a Tris-compatible flat pH probe with a digital control unit connected to the LoggerPro Software (Vernier, Beaverton, OR, USA). All experiments were performed with online pH data acquisition. In some of them, pH was chemically controlled by using an on/off feedback control strategy to impede the decrease of the pH below a set-point. A peristaltic pump with NaOH (3 M) was automatically activated at reaching the lower limit of pH (dead band 0.1). The superior limit was uncontrolled, thus enabling the spontaneous recovery of pH during solventogenesis phase. The pH probes were sterilized by immersing them in 50% (v/v)

ethanol for a minimum of 12 h, after which they were washed with sterile water (Qureshi et al., 2018). The agitation system was that of an AMPTS II device (Bioprocess Control, Sweden). The fermentation system was connected to a gas washing bottle with a water-lock containing water or NaOH (3 M). Additionally, the set-up was coupled to an automatic sampling system. Samples were taken at desired fermentation points to determine concentrations of cells, monosaccharides, and products (volatile fatty acids and solvents).

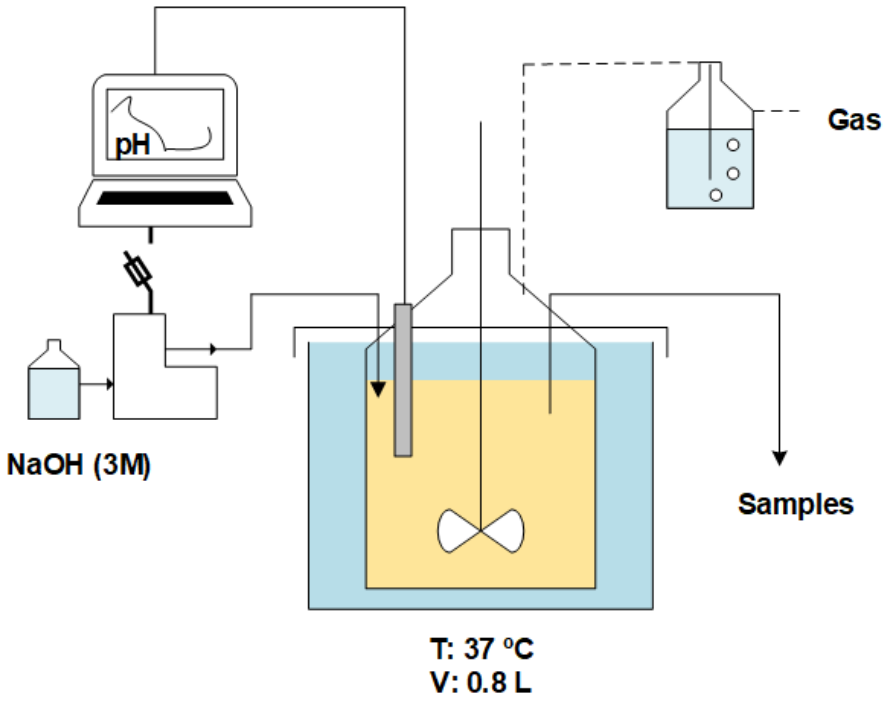

Figure 5.2. Experimental set-up of batch mode for ABE fermentation.

The monosaccharide solutions were sterilized independently by autoclaving (121 ºC, 10 minutes, MED20, JPSelecta, Spain). The metal solution was filter-sterilised by 0.22 μm. When $CaCO_3$ was used as buffer it was sterilized inside the reactor without any liquid present. The rest of the medium components were autoclaved jointly (buffer and nutrient solution, 121 ºC, 20 minutes, MED20, JPSelecta, Spain). After mixing the solutions in the reactor, it was flushed with N_2 for 30 minutes prior the inoculation with 5% (v/v) active cells of *C. acetobutylicum* DSM 792. All batch fermentations were carried out at 37 ºC and 120 rpm. The incubation time was between 48 to 192 h depending on the assay.

Throughout this PhD thesis, several modifications in the fermentation media have been tested using as a base formulation that showed in Table 5.4. The modified P2

media of Table 5.4 was based on the developed by Monot et al. (1982). Changes in this PhD thesis referred to modifications in the monomeric sugar concentration and/or composition and in the buffer formulation. Carbon sources included: glucose (33; 60; 66 and 100 g L^{-1}); xylose (60 g L^{-1}); several mixtures of glucose:xylose (45:15; 30:30 and 35:15 g L^{-1}) and rice straw hydrolysate. Among buffer formulation, ammonium acetate (2.2 g L^{-1}) was exchanged to $CaCO_3$ (2.5 o 5 g L^{-1}) in some experiments. In these cases, inorganic nitrogen was supplied with NH_4Cl (2 g L^{-1}).

Table 5.4. Modified P2 fermentation medium for ABE fermentation with *C. acetobutylicum*.

Component	**Concentration (g L^{-1})**	
Pure sugars (glucose and/or xylose) or rice straw hydrolysate	Variable	Monosaccharide solution
Yeast extract	5.0	Buffer and nutrient solution
Ammonium acetate	2.2	
K_2HPO_4	0.5	
KH_2PO_4	0.5	
Resazurin sodium salt	0.001	
Antifoam 204	0.01%	
$MgSO_4 \cdot 7H2O$	0.2	Metal solution
$MnSO_4 \cdot 7H2O$	0.01	
$FeSO_4 \cdot 7H2O$	0.05	

5.5.1.1. Co-culture experiments

Co-culture experiments are reported in Chapter 8, the inoculation of *S. cerevisiae* was delayed from the inoculation of *C. acetobutylicum*. For these experiments, serum bottles (50 mL) were used as a screening system for stablishing the elapsed time of inoculation between both microorganisms with the aim to promote butanol over ethanol production. The first microorganism to be grown was *C. acetobutylicum* after which *S. cerevisiae* was inoculated with 0, 5 and 10 h of difference. The modified fermentation medium P2 (Table 5.4) with 35 g L^{-1} of glucose and 15 g L^{-1} of xylose was used in a total volume of 40 mL. Both microorganisms were inoculated by using 5% (v/v) of active cells. Growth was carried out at 37 ºC and 150 rpm during 48 h (Stuart Si500, Cole-Palmer, USA). Next, the batch fermenter (0.8 L) with the monitoring and controlling system was employed for developing the co-cultures assays with synthetic media and rice straw hydrolysate.

5.5.2. Continuous stirred tank reactor (CSTR)

5.5.2.1. ABE fermentation

A CSTR with immobilised biomass in plastic carriers was used for the experiments described in Chapter 7. The assay was carried out using 0.075 L of working volume in a 0.25 L total volume reactor. The experimental set-up is shown in Figure 5.3. Same online pH data acquisition and control was used as indicated in Section 5.5.1. A multichannel peristaltic pump (Ismatec Reglo ICC, Cole-Parmer, USA) was used to regulate identical in-and-out liquid flow rates. The gas outlet was coupled to a gas washing bottle with a water-lock. Anaerobic conditions were obtained by flushing N_2 for 10 minutes prior inoculation. The fermentation started with a 5% inoculum (v/v) at 37 ºC and 150 rpm. The temperature was kept constant by a water heating coil. The mixing was accomplished with a magnetic stirrer. Sampling was done from the effluent.

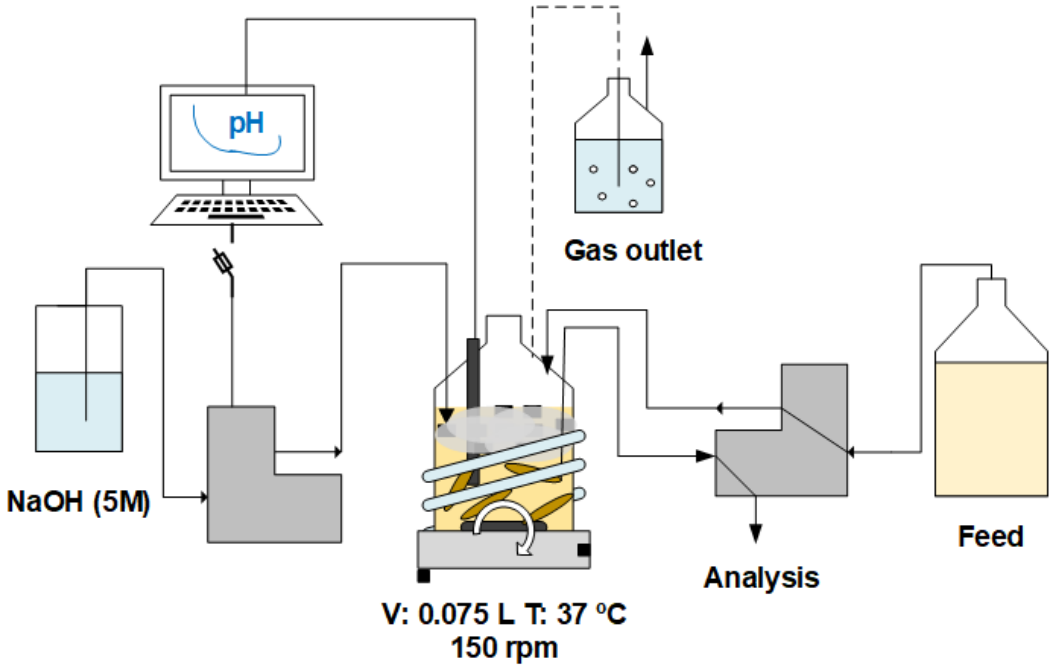

Figure 5.3. Experimental set-up for the immobilised continuous ABE fermentation.

Polypropylene plastic rings (Refill-tech PP rings, Refill-tech Solutions, Italy) were employed as packing material at 7.5% (w/v). The physical properties are summarized in Table 5.5. The packing material was sterilised inside the reactor without any liquid by autoclaving (121 ºC, 20 minutes). Prior the fresh media feeding, anaerobic conditions in the feeding tank were accomplished by insufflating N_2 during 10 minutes. Anaerobic conditions were kept by connecting a Tedlar bag (FlexFoil Sample Bag, SKC, United Kingdom) filled with N_2 to the feeding tank.

Table 5.5. Physical properties of the polypropylene plastic rings.

Carrier	Density (kg m^{-3})	Specific surface (m^2 m^{-3})	Void index (%)
Plastic rings (Refill-tech)	110.7[1]	348[1]	91[1]

[1]Information supplied by the manufacturer.

The conditions tested are listed in Table 5.6. The operation was carried out by using two set-points for minimum pH (6.0 and 4.8). The fermentation media of the batch assays was also used herein (buffer, nutrients, and metal solutions, Table 5.4). Monomeric sugar (glucose, 60 g L^{-1}) was employed as model substrate for stablishing the best dilution rate. Glucose was fed during 216 h varying the dilution rate (h^{-1}) from 0.042 to 0.333 h^{-1}. In the first stages up to a dilution rate of 0.167 h^{-1}, minimum pH was set-up at 6.0 to favour biomass growth and volatile fatty acid production in order to promote biofilm formation. At 144 h (dilution rate of 0.167 h^{-1}), solventogenesis was enhanced by decreasing the set-point of minimum pH to 4.8. In the last stage, glucose was switched to a synthetic formulation of glucose:xylose mixture (40:20 g L^{-1}) that mimics rice straw hydrolysate composition.

Table 5.6. Experimental conditions for the immobilized continuous ABE fermentation.

Time (h)	pH set-point	Carbon source (g L^{-1})	Dilution rate (h^{-1})
0-30	6.0	Glucose (60)	Batch
30-72	6.0	Glucose (60)	0.042
72-121	6.0	Glucose (60)	0.083
121-144	6.0	Glucose (60)	0.167
144-192	4.8	Glucose (60)	0.167
192-216	4.8	Glucose (60)	0.333
216-288	4.8	Glucose:xylose (40:20)	0.333

5.5.2.2. HBE fermentation

A CSTR was used for performing the HBE fermentation with CO by *C. carboxidivorans* (Results in Chapter 9). Assays were carried out using 0.12 L of working volume in a 0.25 L total volume in MiniBio bioreactors coupled with an Applikon my-Control unit (Applikon Biotechnology, Delft, Netherlands). The experimental set-up is represented in Figure 5.4. The media composition for *C. carboxidivorans* P7 was composed by yeast extract (1 g L^{-1}); mineral solution (25 mL L^{-1}, Table 5.7); trace metal solution (10 mL L^{-1}, Table 5.8); vitamin solution (10 mL L^{-1}, Table 5.9) and L-cysteine (0.6 g L^{-1}). The fermentation media was sterilised by autoclaving (121 ºC, 20 minutes, NC 40M, Nüve, Turkey) except for the vitamin solution and L-cysteine. Vitamin solution

was filter-sterilised by 0.22 µm and L-cysteine was autoclaved separately (121 ºC, 10 minutes, NC 40M, Nüve, Turkey). Anaerobiosis was accomplished by insufflating pure CO at 10 mL min^{-1} prior 24 h of inoculation. CO flux was kept constant until the end of the experiment. Before the inoculation with *C. carboxidivorans,* vitamin solution and L-cysteine were added to the reactor. Then, the reaction started by inoculation at an OD_{600} of 0.05 of active cells. Fermentations were developed at 35 ºC and 250 rpm. The liquid feed addition and pH control began after ~24 h of inoculation. The pH was constantly controlled at a pre-stablished set-point by automatic dosing pumps connected to NaOH and HCl (0.5 M, each one). The dilution rate was controlled by the feeding pump. The feed liquid was prepared identically to the fermentation medium. The anaerobiosis of the liquid feed was accomplished by insufflating N_2. The volume of the reactor was controlled by an outlet conduction at the level of liquid connected to a peristaltic pump.

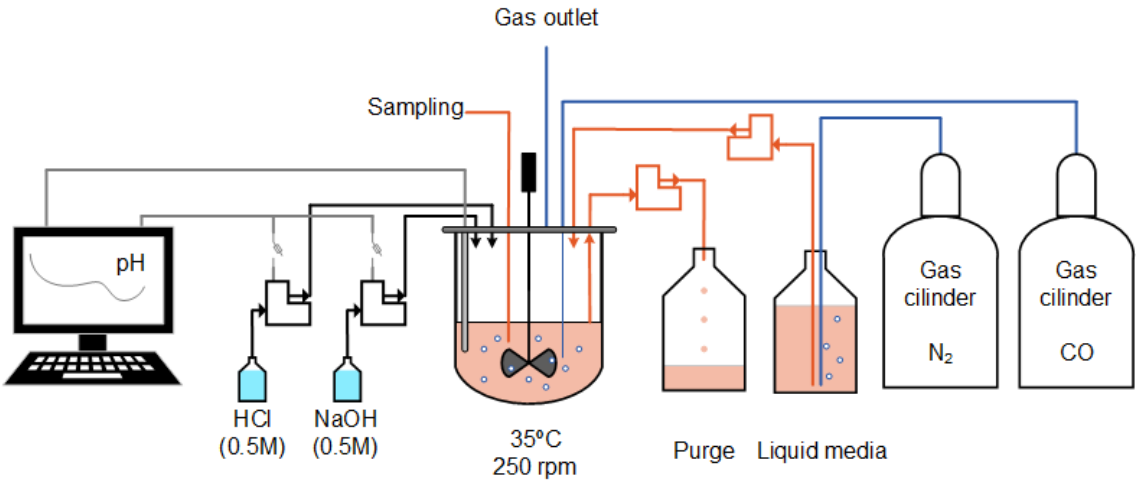

Figure 5.4. Experimental set-up of the CSTR for HBE fermentation.

Table 5.7. Composition of mineral stock solution for HBE fermentation.

Component	**Concentration (g L^{-1})**
Sodium chloride	80.0
Ammonium chloride	100.0
Potassium chloride	10.0
Monopotassium phosphate	10.0
Magnesium sulphate	20.0
Calcium chloride	4.0

Table 5.8. Composition of the trace metal stock solution for HBE fermentation.

Component	Concentration (g L^{-1})	Component	Concentration (mg L^{-1})
Nitrilotriacetic acid	2.0	Cupric chloride	20.0
Manganese sulphate	1.0	Nickel chloride	20.0
Ferrous ammonium sulphate	0.8	Sodium molybdate	20.0
Cobalt chloride	0.2	Sodium selenate	20.0
Zinc sulphate	0.2	Sodium tungstate	20.0

Table 5.9. Composition of vitamin stock solution for HBE fermentation.

Component	Concentration (mg L^{-1})
Pyridoxine	10.0
Calcium pantothenate	5.0
Thiamine	5.0
Riboflavin	5.0
Thioctic acid	5.0
Nicotinic acid	5.0
Para-amino benzoic acid	5.0
Vitamin B12	5.0
D-biotin	2.0
2-mercaptoethane-sulphonic acid	2.0
Folic acid	2.0

5.6. Analytical methods

The reactors were monitored by the measurement of suspended biomass, monosaccharides, acids and solvents. Samples were taken at appropriates time-points for each experiment.

5.6.1. Suspended biomass

Suspended biomass was followed by measuring the optical density at 600nm (OD_{600}) using UV-vis spectrophotometers. For the experiments with *C. acetobutylicum* (Chapters 6, 7 and 8) an SpectroFlex 6600 (WTW, Germany) was used. The experiments with *C. carboxidivorans* (Chapter 9) were analysed with an SPECORD 50 UV-VIS (Analytic Jena, Germany). Cell dry weight (gDM L^{-1}) was estimated from calibration curves (Table 5.10).

Table 5.10. Relationship between the dry cell weight and the OD_{600} of the microorganisms employed in this PhD thesis.

Microorganism	Equation
C. acetobutylicum DSM 792	$gDM\ L^{-1} = 0.2941 \cdot OD_{600} + 0.0331$
S. cerevisiae EYS4	$gDM\ L^{-1} = 0.1885 \cdot OD_{600} + 0.0447$
C. carboxidivorans DSM 15243	$gDM\ L^{-1} = 0.4 \cdot OD_{600}$

5.6.2. Quantification of compounds of interest

5.6.2.1. ABE fermentation

For ABE fermentation, glucose, xylose, arabinose, cellobiose, acetic acid, butyric acid, ethanol, acetone, and butanol were quantified. Inhibitory compounds (furfural, 5-HMF, levulinic acid and phenolic compounds) were also determined when employing the rice straw hydrolysate. Samples were centrifuged (10000 rpm, 5 minutes) and filtered by 0.22 µm polyvinylidene fluoride membrane (Labbox, Spain). The determination of dissolved metabolites in the fermentation media was carried out with the following analytical techniques.

- Ionic chromatography (IC)

The quantification of glucose for experiments described in Chapter 6 was carried out with an Ionic Chromatograph 883 Basic IC plus (Metrohm, Switzerland) equipped with an amperometric detector (945 Professional Detector Vario, Metrohm, Switzerland) at 35 ºC. The column was a Metrosep Carb 2 2 – 150/4.0 (Metrohm, Switzerland) with NaOH (100 mM) and sodium acetate (3 mM) as eluent. The flow was set at 0.5 mL min^{-1}.

- Gas chromatography (GC)

The quantification of acetic acid, butyric acid, butanol, acetone and ethanol for experiments described in Chapter 6 was carried out with a gas chromatograph 7890A GC-System (Agilent Technologies, USA). The equipment was coupled with a flame ionization detector (FID) and a Rtx-VMS column (30 m x 0.25 mm ID, Restek, USA). The carrier gas was helium (99.99%, *Carburos Metálicos*, Spain) at 1.1 mL min^{-1} of flow rate. The injection volume was 1 µL with a split ratio of 1:5. The oven ramp was 100 ºC for 7 minutes, 30 ºC/min until 210 ºC and 210 ºC for 5 minutes. The injector temperature was set at 190 ºC and the detector temperature at 240 ºC.

- High performance liquid chromatography (HPLC)

For the experiments reported in Chapters 7 and 8, samples were analysed using an HPLC 1100 Series (Agilent Technologies, Santa Clara, CA, USA). The column employed was an Aminex® HPX-87H (300 mm x 7.8 mm, Bio-Rad Laboratories Inc., USA) operated at 50 ºC. The mobile phase was H_2SO_4 (5 mM) with a flow 0.6 mL min^{-1}. The system was equipped with a refractive index detector (RID) operated at 35 ºC and a diode array detector (DAD). The RID was employed to quantify glucose, xylose, arabinose, cellobiose, butanol and ethanol. The DAD was used at 210 nm to detect acetic, butyric and levulinic acids and at 280 nm to detect acetone, furfural and 5-HMF.

- Phenolic compounds

The quantification of total phenolic compounds from the rice straw hydrolysate was determined by the colorimetric Folin Denis methodology (Folin and Denis, 1912).

5.6.2.2. HBE fermentation

For HBE fermentation (Chapter 9), acetic acid, butyric acid, hexanoic acid, ethanol, butanol and hexanol were quantified. Liquid samples from the fermentation media were previously centrifuged (13000 rpm, 10 minutes, Centrifuge MiniSpin, Eppendorf, Italy) to remove cells and suspended particles. The fermentation metabolites were determined with an HPLC (HP1100, Agilent Co., EEUU) equipped with a Rezex™ ROA-Organic Acid H+ column (8%, 300 x 7.8 mm, Phenomenex, Italy) and a RID. The column temperature was set at 60ºC and the mobile phase was H_2SO_4 (3.5 mM) at 1.0 mL min^{-1}.

5.6.3. Equations

Maximum growth rates of batch experiments were estimated during the exponential growth phase using Equation 5.1:

$$\mu_{max} = \frac{ln(X_{t2}) - ln(X_{t1})}{t_2 - t_1} \quad (5.1)$$

where the μ_{max} is the maximum growth rate (h^{-1}); X_{t2} and X_{t1} are the biomass concentration at the starting and ending point of the exponential growth phase (g L^{-1}), and t_2 and t_1 are the times as the starting and ending of the exponential growth phase (h).

Maximum consumption and production rates of batch experiments were calculated at their exponential phase for each metabolite using Equation 5.2:

$$r = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \quad (5.2)$$

where the *r* is the maximum consumption and production rates (g L^{-1} h^{-1}); S_{t2} and S_{t1} are the monosaccharide or species concentration at the starting and ending point of the exponential growth phase (g L^{-1}), and t_2 and t_1 are the times as the starting and ending of the exponential growth phase (h).

The estimation of undissociated acetic or butyric acid concentration was determined by using the Henderson-Hasselbalch equation (Equation 5.3) and Equation 5.4:

$$pH = pK_a + log\frac{[A^-]}{[HA]} \quad (5.3)$$

$$[Acid] = [A^-] + [HA] \quad (5.4)$$

in which pK_a of acetic and butyric acids are 4.76 and 4.82; *[Acid]* corresponds to the quantified acid concentration (g L^{-1}) and *[A^-]* and *[HA]* to the dissociated and undissociated acid concentrations (g L^{-1}), respectively.

The productivity in CSTR was estimated by Equation 5.5:

$$P_s = S \cdot D \quad (5.5)$$

where P_s is the productivity (g L^{-1} h^{-1}); *S* is the species concentration (g L^{-1}) and *D* is the dilution factor (h^{-1}).

5.7. References

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6. THE COMBINED EFFECT ON INITIAL GLUCOSE CONCENTRATION AND PH CONTROL STRATEGIES FOR ACETONE-BUTANOL-ETHANOL (ABE) FERMENTATION BY *CLOSTRIDIUM ACETOBUTYLICUM* DSM 792

The butanol is a widely applicable molecule in the biofuel and chemical industries. Hence, shifting from the petrochemical production of butanol to a more sustainable production from lignocellulosic feedstocks is necessary. Solventogenic Clostridium can metabolize monosaccharides from lignocellulosic hydrolysates producing biobutanol via the ABE fermentation. However, the performance of ABE fermentation to produce biobutanol by C. acetobutylicum DSM 792 is highly dependent on the carbon source composition and the pH of the fermentation media. The motivation of this chapter was to evaluate the combined effect of pH regulation with a series of initial glucose levels. These different initial glucose concentrations would help to understand the ABE fermentation by C. acetobutylicum on lignocellulosic hydrolysates.

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THE COMBINED EFFECT ON INITIAL GLUCOSE CONCENTRATION AND PH CONTROL STRATEGIES FOR ACETONE-BUTANOL-ETHANOL (ABE) FERMENTATION BY *CLOSTRIDIUM ACETOBUTYLICUM* DSM 792

Abstract

The use and depletion of fossil fuels raised the interest in biofuels like biobutanol. *Clostridium acetobutylicum* DSM 792 is capable of producing biobutanol through ABE fermentation. Butanol production can be influenced by low sugar concentrations, like those obtained after hydrolysis of pre-treated lignocellulosic biomass. This study aimed to evaluate the influence of the initial glucose concentrations (33, 66 and 100 g L^{-1}) and pH control strategies on biobutanol production and glucose consumption. Uncontrolled pH fermentation exhibited low butanol production due to either glucose exhaustion (33 g L^{-1}) or the phenomenon of acid crash (66 and 100 g L^{-1}), which was alleviated by the use of any of the minimum pH set-points (4.8; 5.0; 5.1 and 5.5). Fermentation at a pH_{min} of 5.1 gave the best performance in butanol production and glucose consumption rate. Fermenting with a pH_{min} of 5.1 with 33 g L^{-1} of initial glucose caused acidogenic fermentation, while the use of 66 and 100 g L^{-1} of glucose led to a ~1.5 and ~1.7-fold increase in butanol concentration over their counterparts without pH control respectively. By controlling the pH after the acidogenic phase 15.8 g L^{-1} of butanol was obtained with 100 g L^{-1} of glucose, which was ~2-fold higher than without pH control. Different initial glucose concentrations therefore require different pH strategies to optimize butanol production.

Highlights

- Proper pH control strategy depended on substrate concentration for *C. acetobutylicum*.
- Control of pH≥5.1 allowed to overcome acid crash for glucose >60 g L^{-1}.
- The minimum pH control improved ~1.7-fold ABE at glucose >60 g L^{-1}.
- Initial glucose of 33 g L^{-1} led to poor ABE production with or without pH control.

Keywords: ABE fermentation; Acid Crash; Biobutanol; *Clostridium acetobutylicum*; Glucose; pH control.

6.1. Introduction

The extensive use of fossil fuels has led to a series of problems, such as climate change, an energy crisis and health problems. The importance of these issues has revived the interest of governments in the production of greener renewable energy. Biofuels generated biologically will become viable alternatives to oil and other derivate products when the price of biofuels becomes competitive (Pugazhendhi et al., 2019). Several types of biofuel have recently gained interest, including biomethanol, bioethanol and biobutanol. Of these, biobutanol appears especially likely to become widely used due to its physicochemical properties (Dürre, 2007). In addition, biobutanol has a higher heating value and lower vapour pressure than bioethanol and can be transported and distributed via existing pipelines and service stations reducing the investment cost in infrastructures (Bharathiraja et al., 2017; Ibrahim et al., 2017).

Solventogenic Clostridium strains can produce biobutanol by Acetone-Butanol-Ethanol (ABE) fermentation. These *Clostridium* strains (*Clostridium acetobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium beijerinckii* or *Clostridium sporogenes*) metabolize monosaccharides (pentoses and hexoses) from different sources, such as starch or lignocellulosic biomass to produce ABE (Gottumukkala et al., 2013; Moon et al., 2016). Among the raw materials currently available for ABE production, lignocellulosic biomass appears as an ideal candidate in terms of the circular economy, as it can be obtained from a wide range of residues such as rice straw, sweet sorghum stalk or sago hampas. Unlike the first generation of biofuels obtained from feedstocks (sugarcane, starch, molasses, etc.), lignocellulosic biomass does not interfere with the food chain supply (Prasad et al., 2019).

Lignocellulosic biomass is composed mainly of cellulose, hemicellulose and lignin. However, it requires a pre-treatment and enzymatic hydrolysis to release the fermentable sugars prior to fermentation, which affects the final composition. Researchers have recently applied different pre-treatments (acid, alkali, ammonium sulphide, organic solvents or ionic liquids) to diverse lignocellulosic materials (softwood, apple pomace or rice and wheat straw) resulting in a range of 22 to 52 g L^{-1} of total sugars after hydrolysis depending on their conditions (Gottumukkala et al., 2013; Jin et al., 2019; Qi et al., 2019; Trinh et al., 2018; Valles et al., 2020). Unfortunately, low sugar concentrations can result in low butanol production, such as ~2 g L^{-1} of butanol with 20 g L^{-1} of glucose (Ibrahim et al., 2015). Novel strategies have been developed to concentrate the lignocellulosic hydrolysate, including evaporation or vacuum. However,

these approximations increase the pretreatment cost (Lu et al., 2012; Survase et al., 2019).

ABE fermentation is characterised by two phases: an acidogenic phase followed by a solventogenic phase. In the acidogenic phase, biomass, acids (butyric and acetic) and gases (CO_2 and H_2) are produced, accompanied by a drop in pH. During the solventogenic phase, the acids are re-assimilated producing butanol, acetone and ethanol with pH recovery (Monot et al., 1982). The bacteria selected and the external conditions, such as medium composition, temperature and pH, regulate the end products obtained (Cheng et al., 2012; Díez-antolínez et al., 2016). *C. acetobutylicum* is a widely-known bacterium used for ABE fermentation (Bahl et al., 1982; Mirfakhar et al., 2020; Qureshi et al., 2001), although there are still some issues that should be evaluated for efficient production of solvents (Luo et al., 2020). In this regard, proper pH control is a key factor in ABE fermentation. High pH in the medium is related to more biomass and acids concentration in the reactors (Raganati et al., 2016; Yang et al., 2013), while the reduced pH due to acid production is needed to produce solvents (Li et al., 2011). In fact, a minimum undissociated butyric acid concentration is necessary to initiate solvent production (Monot et al., 1984). Moreover, the relation of the dissociated and undissociated species of acids, which are directly linked to the pH, plays an important role in the fermentation process (Yang et al., 2013). Excessive acid production and low pH can create the conditions to cause acid crash as the undissociated acid concentration rises. As a consequence, acid crash causes the cease of glucose consumption and butanol production resulting in an ABE production failure (Maddox et al., 2000).

Chemical control is now being applied in ABE fermentation, especially in continuous reactors, as a way of regulating the medium pH (Millat et al., 2013). The pH regulation allows the study of the effect of the substrate on the butanol production. For example, butanol production was ~2.3-fold higher when xylose was used instead of lactose by keeping the pH controlled at 4.7 under continuous ABE fermentation with *C. acetobutylicum* DSM 792 (Procentese et al., 2015a, 2015b). In batch mode, Guo et al. (2012) demonstrated that the best pH set-point range was between 4.5 and 4.9 when fermenting 60 g L^{-1} of glucose and *C. acetobutylicum* XY16. Alternatively, by implementing pH control exclusively during the acidogenic phase Luo et al. (2019). obtained ~8.1-fold higher butanol production by avoiding acid crash with 60 g L^{-1} of glucose and *C. acetobutylicum* ATCC 824. With this pH control strategy these authors reported an increase in butanol production from 9.42 to 11.52 g L^{-1} by adding trace concentrations of inhibitors such as the phenolic compounds. Implementing of a pH

control strategy would thus be beneficial for improving ABE productivity from lignocellulosic hydrolysates. Despite this, more systematic studies are required on ABE fermentation to evaluate the combined effect of the initial substrate concentration and the pH control strategy.

The aim of this study was thus to systematically evaluate the combined effect of the initial sugar concentration and the pH control strategy in ABE fermentation by *C. acetobutylicum* DSM 792 using batch reactors and glucose as the model substrate. For this, we compared the performance of the process with three different levels of initial glucose concentration (33, 66 and 100 g L^{-1}) either without pH control or with two pH control strategies: firstly, by limiting the minimum pH over the fermentation time course, and secondly by spontaneous acidogenesis without pH control followed by limiting the minimum pH during solventogenesis.

6.2. Material and methods

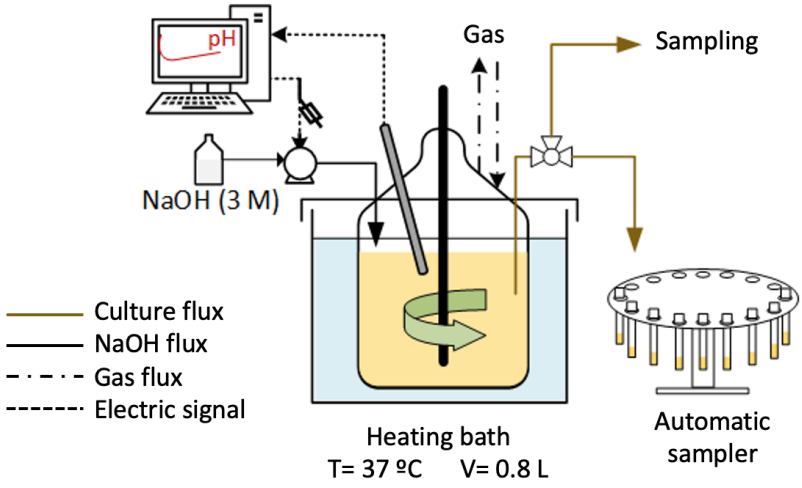
6.2.1. Microorganism and chemical reagents

C. acetobutylicum DSM 792 was obtained from DSMZ, Germany (German Collection of Microorganisms and Cell Cultures). The cultures were maintained in 20% glycerol at -80 ºC. Prior to the experiments, the stock was cultured statically at 37 ºC in 19 g L^{-1} of Reinforced Clostridial Medium (RCM) supplemented with 10 g L^{-1} of glucose as a seed inoculum. Chemicals were purchased from VWR, except for antifoam 204 (Sigma-Aldrich), $CaCO_3$ (Merk) and yeast extract (Alfa Aesar).

6.2.2. Experimental set-up

The fermentations were carried out in a modified 1 L bottle (total volume: 1.1 L) with a working volume of 0.8 L (stirred tank reactor, STR). The medium contained (g L^{-1}): glucose, 60 (Solution I); Yeast Extract, 5; NH_4Cl, 2; KH_2PO_4, 0.5; K_2HPO_4, 0.5; Antifoam 204, 0.01 %; resazurin sodium salt, 0.001 (Solution II); $MgSO_4.7H_2O$, 0.2; $MnSO_4.7H_2O$, 0.01; $FeSO_4.7H_2O$, 0.05 (Solution III) and $CaCO_3$, 5. All the components were sterilized prior to inoculation. Solutions I and II were autoclaved at 121 ºC for 10 and 20 min, respectively. Solution III was filter-sterilized by 0.22 μm. $CaCO_3$ was autoclaved (121 ºC, 20 min) inside the reactor without any liquid. The media was flushed with nitrogen for 30-35 min before inoculation to create anaerobiosis. The fermentation was carried out at 37 ºC and 120 rpm with an inoculum size of 5%. The fermentations were monitored by a Tris-compatible flat pH sensor with LoggerPro software (Vernier, USA). When needed, the pH was controlled with NaOH (3M). The pH probes were sterilized with 50% ethanol

solution for at least 12 h. Figure 6.1 shows the experimental setup for the fermentation. Liquid samples from the fermentations were taken at appropriate time-points for the analysis of cell growth, glucose and products (butyric acid, acetic acid, butanol, acetone and ethanol).



Run	glucose, g L^{-1}	pH control	set-point
1	33, 66, 100	No	
2	66	Yes	pH ≥ 4.8, 5.0, 5.1, 5.5
3	33, 66, 100	Yes	pH ≥ 5.1
4	33, 66, 100	Yes	pH ≥ 5.1 from ~24 h

Figure 6.1. Experimental setup and plan for ABE fermentations.

6.2.3. Experimental plan

Four sets (runs) of experiments were planned (Figure 6.1) to study the behaviour of *C. acetobutylicum*. Run 1 was buffered media with 5 g L^{-1} of $CaCO_3$ without active pH control. The effect of three different initial glucose concentrations (33, 66 and 100 g L^{-1}) was evaluated in terms of butanol production and yield. A pH control strategy was applied in the following runs (run 2, 3 and 4). The pH control strategy for run 2 and run 3 consisted of natural acidification by acid production until a minimum pH set-point was reached, after which the pH was not allowed to fall by automatically adding NaOH solution. The base dosing pump was activated as many times as needed by the pH probe readings until the pH value increased 0.1 of the set-point. The spontaneous rise of pH was not controlled, allowing spontaneous pH recovery as the solventogenesis stage advanced.

Run 2 was performed as the screening of several minimum pHs (4.8, 5.0, 5.1 and 5.5) to find the best pH for butanol production and glucose consumption with the intermediate initial glucose concentration (66 g L^{-1}). From these results, a pH of 5.1 was selected to evaluate the ABE fermentation profiles of the three initial glucose concentrations (33, 66 and 100 g L^{-1}) in run 3. A hybrid pH strategy with the three levels of initial glucose concentrations was then carried out in run 4, which consisted of natural acidification up to the spontaneous minimum pH from the acidogenesis stage, followed by a sudden increase of pH to the set-point (5.1). The pH set-point was kept as the minimum value by pH control until the end of the fermentation.

6.2.4. Analytical methods

Biomass concentration (gDM L^{-1}) was calculated from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany) as gDM L^{-1} = 0.2294·OD_{600} (n=8, R^2=0.992). The liquid samples were centrifuged at 10000 rpm for 5 min and filtered by 0.22 µm for glucose and products analysis. Glucose was analysed using an ionic chromatograph (Basic IC Plus 833, Metrohm, Switzerland) with an amperometric detector (945 Professional Detector Vario IC Amperometric Detector, Metrohm, Switzerland). The eluent was 100 mM NaOH and 3 mM sodium acetate with a flow of 0.5 ml min^{-1} and the temperature detector was set at 35 ºC. Fermentation products were analysed by a gas chromatograph (7890A GC-System, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and an Rtx-VMS column (Restek, USA), using helium as the carrier gas with a flow rate of 1.1 mL min^{-1}. The oven programme was: 100 ºC for 7 min; 30 ºC/min until 210 and 210 ºC for 5 min. The temperatures of the injector and the detector were set at 190 ºC and 240 ºC, respectively. Cell growth was measured by an UV-Vis spectrophotometer (SpectroFlex 6600, WTW) at 600 nm. Undissociated acid concentration was calculated by the Henderson-Hasselbalch Equation and the pKa of acetic acid (4.76) and butyric acid (4.82).

6.2.5. Determination of the glucose consumption rate and the specific growth rate

Glucose consumption and maximum growth rates were calculated during the exponential growth phase by Equations 6.1 and 6.2:

$$-q_{glucose} = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \quad (6.1)$$

$$\mu_{max} = \frac{ln(X_{t2}) - ln(X_{t1})}{t_2 - t_1} \quad (6.2)$$

where $-q_{glucose}$ is the glucose consumption rate (g L^{-1} h^{-1}), S_{t1} and S_{t2} are the glucose concentrations at the beginning and end of the exponential growth phase (g L^{-1}), t_2 and t_1 are the initial and end time of the exponential growth phase (h), μ_{max} is the specific growth rate (h^{-1}), X_{t1} and X_{t2} are the biomass concentrations at the beginning and end of the exponential growth (gDM L^{-1}).

6.3. Results and discussion

6.3.1. $CaCO_3$ buffered ABE fermentation (uncontrolled pH)

Three different initial glucose concentrations (33, 66 and 100 g L^{-1}) were used as the substrate for the ABE fermentation under 5 g L^{-1} $CaCO_3$ as a buffering agent. These initial sugar concentrations were chosen within the typical range of sugar production after pre-treatment and enzymatic hydrolysis of lignocellulosic biomass. Sugar concentrations below ~45 g L^{-1} are usually obtained, although concentrations techniques may be applied to increase them (Ibrahim et al., 2017). The fermentation profiles are shown in Figure 6.2. The $CaCO_3$ was able to buffer the fermentation media and keep the minimum pH around 4.6 in all the experiments. The maximum production of acetic and butyric acids was between 5 and 6 g L^{-1} in all the tests. In fact, high acid concentration can be related to the presence of $CaCO_3$. Ren et al. (2010) obtained ~6-fold more acetic and ~1.4-fold more butyric when culturing *C. acetobutylicum* ATCC 824 in P2 media supplemented with $CaCO_3$ than supplementing with P2 media only. $CaCO_3$ could stimulate the acid production by the stabilization of the cell membrane proteins due to the presence of divalent ions (Mg^{+2} and Ca^{+2}) (El Kanouni et al., 1998). The three experiments had almost the same biomass production. The glucose consumption rate and maximum growth rate were nearly the same as the average values of 2.09 ± 0.15 g L^{-1} h^{-1} and 0.197 ± 0.008 h^{-1} respectively. However, the test with the lowest initial glucose experiment (33 g L^{-1}) showed some differences in the ABE fermentation profile and product yields.

In the experiment with 33 g L^{-1} of initial glucose, nearly all the glucose was depleted in the first 24 h, accompanied by increased butanol production (~2.64 g L^{-1} at 28 h). However, the rapid glucose depletion limited, to some extent, the assimilation of butyric acid to butanol, giving a final butanol concentration of 3.35 g L^{-1} (ABE concentration: 3.99 g L^{-1}). Butyric acid remained at a high concentration (~6.0-6.6 g L^{-1}) from 15 h, and glucose depletion kept the butyric acid assimilation rate lower than its

production rate. At the glucose level of 33 g L^{-1}, maximum butanol and ABE yields of 0.099 and 0.118 g g^{-1}, respectively, were obtained (Table 6.1). Similar solvent yields have been reported in previous studies. For example, Ibrahim et al. (2015) obtained a butanol (ABE) yield of 0.11 (0.15) and 0.13 (0.22) with 20 and 40 g L^{-1} of glucose with *C. acetobutylicum* ATCC 824 without pH control. In Long et al. (1984), who tested gradually increasing glucose concentration, the *C. acetobutylicum* P262 strain produced ~1, ~4, ~12 and ~13 g L^{-1} of ABE with 20, 30, 40 and 60 g L^{-1} of glucose, respectively, indicating that >40 g L^{-1} of glucose is required to achieve ABE yields >0.20. However, other *Clostridium* species may behave differently under the same glucose concentrations. For example, *C. beijerinckii* P260 produced 8.0 (11.5) g L^{-1} of butanol (ABE) with 29.3 g L^{-1} of initial glucose concentration without pH control (Jin et al., 2019). Our results are thus important for adequate planning the conversion of butanol from lignocellulosic waste using *C. acetobutylicum*.

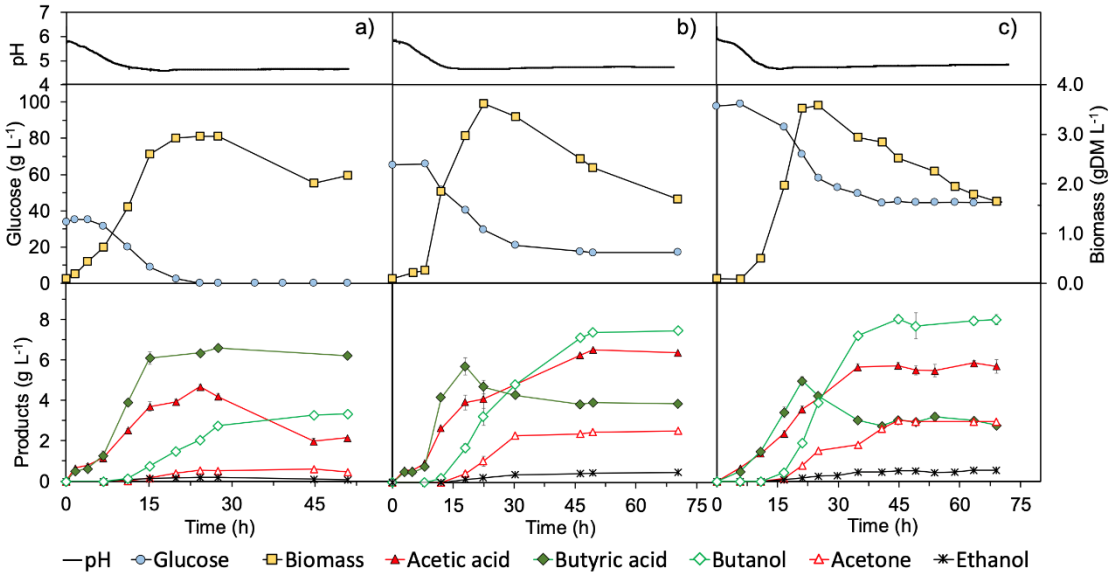


Figure 6.2. Effect of initial glucose concentration on butanol production under uncontrolled pH by *C. acetobutylicum* (a, 33; b, 66; c, 100 g L^{-1} of initial glucose).

However, the reactors with 66 and 100 g L^{-1} achieved butanol (ABE) concentrations of 7.47 (10.50) and 8.01 (11.59) g L^{-1} respectively (Table 6.1). These titers gave a ~2.3 (~2.8)-fold increase in butanol (ABE) production when compared with 33 g L^{-1} of initial glucose concentration. In addition, the butanol (ABE) yields were 0.155 (0.217) and 0.151 (0.220) g g^{-1} for 66 and 100 g L^{-1} respectively (Table 6.1), an increase of ~54% (~85%) versus the 33 g L^{-1} of the initial glucose experiment. A minimum pH of ~4.65 was achieved at ~16-18 h, with an acid production (acetic plus butyric) of 9.63 (at 18 h) for 66 g L^{-1} of initial glucose and 8.54 g L^{-1} (at 21h) for the 100 g L^{-1} reactor. Also,

~2 g L^{-1} of butyric acid was reassimilated in both reactors from the acid peak concentration until the end of the fermentation. The better performance of these two reactors than the lowest initial glucose experiment is associated with the residual glucose remaining when the minimum pH was reached (near the end of the acidogenic stage). A glucose concentration higher than 40 g L^{-1} (at ~16-18 h) was sufficient to develop the solventogenic stage in both reactors. However, the glucose was not completely depleted in either reactor, with only 74% of the glucose consumed in the 66 g L^{-1} experiment and 54% in the 100 g L^{-1}. Glucose consumption sharply declined after 12 h from the point at which the butyric acid peaked Figure 6.2. This could be associated with the high undissociated acid concentrations reached when the acids peaked (63 and 53 mM for 66 and 100 g L^{-1} of initial glucose reactors, respectively). Accordingly to Maddox et al. (2000), acid crash phenomenon can occur when the upper limit of total undissociated acid concentration (57 to 60 mM) is exceeded. Other authors have obtained better butanol production for this strain in similar environmental conditions, but with lower initial glucose concentrations. For example, Yang et al. (2013) reported a butanol production of 10.8 g L^{-1} (butanol yield: 0.216 g g^{-1}) with 50 g L^{-1} of initial glucose with the same minimum pH (4.7) using *C. acetobutylicum* ATCC 824 and 5 g L^{-1} of $CaCO_3$ as a buffer. The lower butanol production and yield obtained in our experiment seems to indicate that the spontaneous ABE fermentation without pH control could facilitate conditions for acid crash when the initial glucose concentrations is higher than 60 g L^{-1}. The undissociated form of the acid is more toxic than the dissociated, as the former species can diffuse through the plasma membrane to the cytosol, where it dissociates and reduces the intracellular pH (Palmqvist and Hahn-Hägerdal, 2000). An active pH control could thus help to limit the concentration of undissociated acid species as a strategy to improve butanol production.

Table 6.1. Production and kinetics parameters under uncontrolled pH (natural evolution of pH). Yields were calculated with the maximum solvent concentration. Glucose conversion refers to the end of fermentation.

$C_{glucose}$ (g L^{-1})	$Butanol_{max}$ (g L^{-1})	ABE_{max} (g L^{-1})	$Y_{B/S}$ (g g^{-1})	$Y_{ABE/S}$ (g g^{-1})	μ_{max} (h^{-1})	$-q_{glucose}$ (g L^{-1} h^{-1})	Glucose conversion (%)
33	3.35	3.99	0.099	0.118	0.188	2.27	100
66	7.47	10.50	0.155	0.217	0.202	2.00	74
100	8.01	11.59	0.151	0.220	0.202	2.02	54

6.4. Fermentations under active pH control

6.4.1. Screening of the set-point for minimum pH

In order to avoid acid crash, a pre-screening experiment was carried out to evaluate the fermentation profiles under different minimum controlled pHs and using 60 g L^{-1} of initial glucose. The minimum pH tested were 4.8; 5.0; 5.1 and 5.5. The chosen pH_{min} control strategy was selected to optimize both butanol production and glucose consumption. The results indicate that controlling the pH_{min} led to reduced total undissociated acid concentration in the reactors. The maximum concentrations (total acetic and butyric acids) were 50.61, 42.03, 37.64 and 39.46 mM for the reactors with the minimum pH of 4.8, 5.0, 5.1 and 5.5 respectively. Controlling the pH thus kept the undissociated acid values below the levels related to acid crash. In addition, complete glucose depletion occurred in all the conditions tested, even though increasing the pH_{min} from 4.8 to 5.5 led to a higher glucose consumption rate, from 1.90 to 3.47 g L^{-1} h^{-1} (Table 6.2). The maximum growth rates were close within any of the pH studied, with an average value of 0.202 ± 0.006 h^{-1}, similar to the reactors with no active pH control (Table 6.1). When pH_{min} was 5.5, the main fermentation product was butyric acid (15.88 g L^{-1}), indicating acidogenic fermentation. In contrast, solventogenesis failed to occur and butanol production was almost residual (2.01 g L^{-1}, yield: 0.031 g g^{-1}) (Table 6.2). In fact, the acidogenic fermentation could have been related to the residual glucose concentration of 6 g L^{-1} at 20 h, which may be below the limit for developing solventogenesis. The reactors at 5.1, 5.0 and 4.8 pH reported similar butanol (ABE) productions of 11.22 (16.12), 11.42 (16.67) and 11.57 (18.27) g L^{-1} respectively. ABE yield was slightly higher at pH of 4.8 (0.301 g g^{-1}) as more ABE was produced. A ~1.5-fold increase in butanol production was obtained over the same initial glucose concentration without pH control. Little data is available in the literature on comparing the effect of different values of pH on *Clostridium* species. Jiang et al. (2014) evaluated a set of pH values from 4.9 to 6.0 for *C. beijerinckii* IB4 with 60 g L^{-1} of initial glucose. This strain showed an optimum pH of 5.5 for butanol production and glucose consumption rate. Other pH may be set for ABE fermentation of other *Clostridium* species. For example, a value of 4.8 was successfully stablished for *C. beijerinckii* DSM 6423 (S. A. Survase et al., 2011) and a value of 5.5 for *C. saccharoperbutylacetonicum* N1-4 (Gao et al., 2016). Our results showed that when pH_{min} was controlled, solvent production and glucose consumption rate outperformed the uncontrolled pH experiment. A pH_{min} of 5.1 was therefore selected for the next experiments as this value showed the highest glucose consumption rate (~3.0 g L^{-1} h^{-1}) among similar butanol titers (~11 g L^{-1}).

Table 6.2. Influence of the minimum controlled pH on production and kinetics parameters. Initial concentration of glucose: 60 g L^{-1}. Yields were calculated with the maximum solvent concentration.

pH	$Butanol_{max}$ (g L^{-1})	ABE_{max} (g L^{-1})	$Y_{B/S}$ (g g^{-1})	$Y_{ABE/S}$ (g g^{-1})	μ_{max} (h^{-1})	$-q_{glucose}$ (g L^{-1} h^{-1})
5.5	2.01	2.92	0.031	0.046	0.204	3.47
5.1	11.22	16.12	0.163	0.235	0.205	3.02
5.0	11.42	16.67	0.179	0.256	0.194	2.61
4.8	11.57	18.27	0.186	0.301	0.206	1.90

6.4.1.1. Combined effect of initial glucose concentration and the minimum pH control

Batch fermentations keeping pH_{min} at 5.1 were performed with glucose concentrations of 33, 66 and 100 g L^{-1} to evaluate the combined effect of controlled pH and the initial glucose concentration on the fermentation process. The profiles of these fermentations are shown in Figure 6.3 and the main representative parameters are given in Table 6.3. The use of a pH control clearly modified the pH profiles as compared to their respective uncontrolled pH fermentations (Figure 6.2). At 5.1, the maximum undissociated acid concentration obtained in the 33, 66 and 100 g L^{-1} of the initial glucose reactors were 43.88; 37.64 and 36.15 mM, respectively, a reduction of about 30-40% in comparison with the non-controlled pH experiments. None of the controlled reactors therefore reached the value of 57-60 mM of total undissociated acids in the media that could cause an acid crash. The maximum biomass concentration in the two reactors with the highest initial glucose concentration (66 and 100 g L^{-1}) were ~1.5-fold higher than the reactor with 33 g L^{-1} of initial glucose. As expected from the pre-screening experiment, active pH_{min} control resulted in higher glucose consumption rate values (average: 3.25 ± 0.21 g L^{-1} h^{-1}) than the uncontrolled reactors (average: 2.09 ± 0.15 g L^{-1} h^{-1}). A similar growth rate was observed in previous experiments with a maximum average growth rate of 0.200 ± 0.009 h^{-1} (Table 6.3). No significant differences were obtained between the three sets of experiments (uncontrolled, pre-screening and pH_{min} at 5.1) (ANOVA p-value: 0.82), indicating that the maximum growth rate value may be used as a checkpoint to determine whether the culture has developed correctly during a sequential series of experiments. In any case, as maximum growth rates are culture dependent they must be determined *in situ*. For example, Liao et al. (2018) and Buendia-Kandia et al. (2018) found values of ~0.18 and ~0.26 h^{-1} respectively, when *C. acetobutylicum* ATCC 824 was grown in different media formulations.

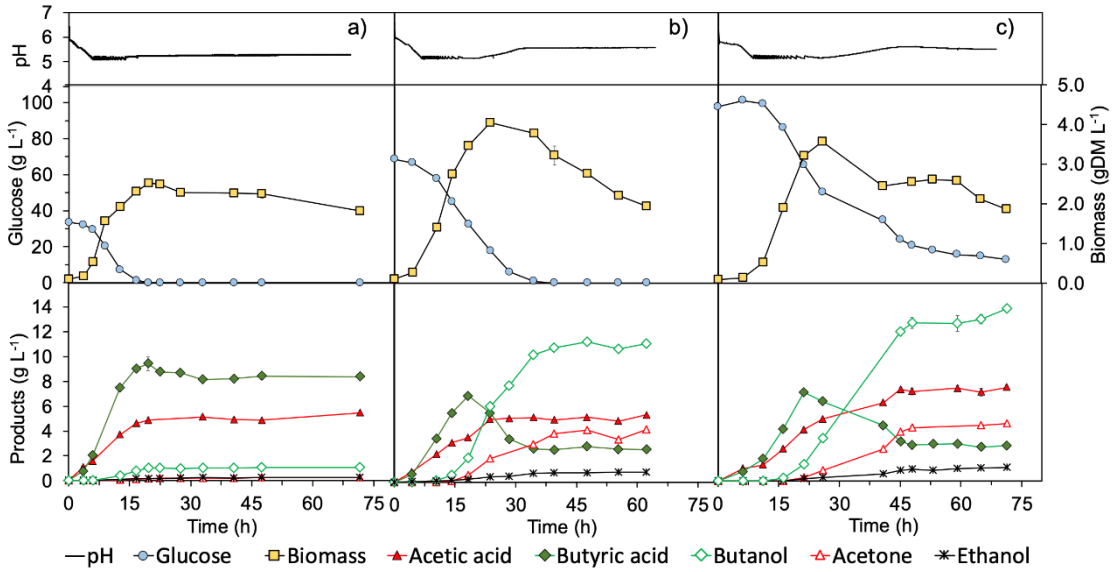


Figure 6.3. Effect of initial glucose concentration on butanol production with pH_{min} at 5.1 by *C. acetobutylicum* (a, 33; b, 66; c, 100 g L^{-1} of initial glucose).

Table 6.3. Production and kinetics parameters under controlled pH (at pH_{min} ≥ 5.1). Yields were calculated with the maximum solvent concentration. Glucose conversion refers to the end of fermentation.

$C_{glucose}$ (g L^{-1})	$Butanol_{max}$ (g L^{-1})	ABE_{max} (g L^{-1})	$Y_{B/S}$ (g g^{-1})	$Y_{ABE/S}$ (g g^{-1})	μ_{max} (h^{-1})	$-q_{glucose}$ (g L^{-1} h^{-1})	Glucose conversion (%)
33	1.09	1.59	0.032	0.047	0.205	3.35	100
66	11.22	16.12	0.163	0.235	0.205	3.02	100
100	13.83	19.55	0.163	0.232	0.190	3.34	87

The ABE fermentation profile of 33 g L^{-1} initial glucose showed a failure in solvent production Figure 6.3. A redistribution of the carbon flux was observed as the main fermentation product was butyric acid (9.43 g L^{-1}) and the glucose was exhausted at ~17 h. In fact, maximum butyric acid concentration increased by ~1.5-fold over the uncontrolled reactor. Butanol (ABE) production was 1.09 (1.59) g L^{-1} and butanol (ABE) yield was 0.032 (0.047) g g^{-1} (Table 6.3). These solvent productions were on average ~0.4-fold less butanol, acetone and ABE concentration than the uncontrolled pH assay, although ethanol production increased from 0.18 to 0.26 g L^{-1}. The yields with 33 g L^{-1} of initial glucose were almost identical to those obtained in the controlled fermentation at pH 5.5, in which the glucose was completely exhausted (Screening pH Section, Table 6.2). The low residual glucose in the early stages of fermentation could explain the failure to switch to solventogenesis and the acidogenic fermentation. *C. acetobutylicum* DSM

792 may not be the most suitable when working at low sugars concentrations, although, Dolejš et al. (2014) were able to produce 2.7 g L^{-1} of butanol with 5.0 pH and 20 g L^{-1} initial glucose concentration using *C. acetobutylicum* DSM 1731.

A minimum glucose concentration may be necessary to properly develop ABE fermentation with pH_{min} at 5.1. Butanol production in the reactors with 66 and 100 g L^{-1} initial glucose was 11.22 and 13.83 g L^{-1}, respectively. These values were a ~1.5 and ~1.7-fold increase over their counterparts with no pH control. Indeed, acetone and ethanol increased an average of ~1.6-fold and ~1.8-fold, respectively, when pH control was applied. This indicates the feasibility of limiting the pH_{min} to 5.1 to improve solvent titers when sufficient carbon source is available for *C. acetobutylicum* DSM 792. When a similar strategy was recently applied by Luo et al. (2019), they obtained 9.42 g L^{-1} of butanol with pH set at 5.0 during acidogenesis using 60 g L^{-1} of initial glucose with *C. acetobutylicum* ATCC 824. Our results confirm that a suitable pH control strategy can increase the butanol titer in ABE fermentation. Butanol and ABE yields were almost identical, with 66 and 100 g L^{-1} of initial glucose concentration (Table 6.3), although more butanol was produced when using 100 g L^{-1} of glucose. In fact, maximum butyric acid was 6.89 g L^{-1} (at ~18 h) for the 66 g L^{-1} reactor and 7.11 g L^{-1} (at ~21 h) for the 100 g L^{-1}, these concentrations represent an average ~1.3-fold increase over uncontrolled pH control fermentations. Furthermore, pH was recovered in both reactors as butyric acid was assimilated. A similar butyric acid concentration of ~2.60-2.80 g L^{-1} remained in both reactors at the fermentation end point, indicating a ~2.1-fold increase in butyric acid reassimilation with pH control over the uncontrolled pH experiment. Complete glucose depletion was achieved in the fermentation with 66 g L^{-1} of initial glucose, while the fermentation with 100 g L^{-1} of initial glucose consumed 84.9 g L^{-1} (87% of total glucose). Therefore, an increase of 42% and 59% in glucose consumption with 66 and 100 g L^{-1} of initial glucose, respectively, was achieved versus the no pH control assays. High initial glucose concentrations may induce substrate inhibition in the *Clostridium* species. For example, Qureshi and Blaschek (2001) found substrate inhibition for *C. beijerinckii* with 158 g L^{-1} of initial glucose. In our study, no substrate inhibition was found at glucose concentrations as high as 100 g L^{-1}, as similar biomass was obtained in reactors with 66 and 100 g L^{-1} of initial glucose concentration. Due to the higher butanol production in the study with 100 g L^{-1} of initial glucose, a concentration step for sugars in lignocellulosic hydrolysates could be a suitable option to enhance the economic viability of ABE fermentation.

6.4.2. Hybrid pH strategy

A hybrid pH control strategy was proposed and tested for the three initial glucose concentrations (33, 66 and 100 g L^{-1}). This strategy consisted of spontaneous pH evolution until reaching minimum pH, followed by a sudden pH step control up to 5.1 at ~21-24 h, and from then on keeping pH_{min} at 5.1. In our reactors with 33 and 66 g L^{-1} of initial glucose, butanol production with the hybrid pH strategy was lower than either uncontrolled pH (2.56 g L^{-1}) or pH_{min} at 5.1 (9.08 g L^{-1}). On the other hand, when higher initial sugars concentrations were used, better fermentation results were found by implementing this pH strategy. This type of pH modification strategy may rely on culture conditions such as growth phase or carbon source availability at the modification time. The results of the fermentation of 100 g L^{-1} of initial glucose under the hybrid pH control strategy are shown in Figure 6.4. The butanol and ABE production for the assay with 100 g L^{-1} of initial glucose was 15.80 and 23.68 g L^{-1} respectively. An increase of ~1.1 and ~1.2-fold in butanol and ABE production over to the experiment carried out at 5.1 pH_{min} with the same initial glucose. Although butanol inhibition could occur when more than 14 g L^{-1} were present in the media when using *C. acetobutylicum* ATCC 824 (Gao et al., 2012), our data indicate that a delay in cell death may allow this limit to be exceeded.

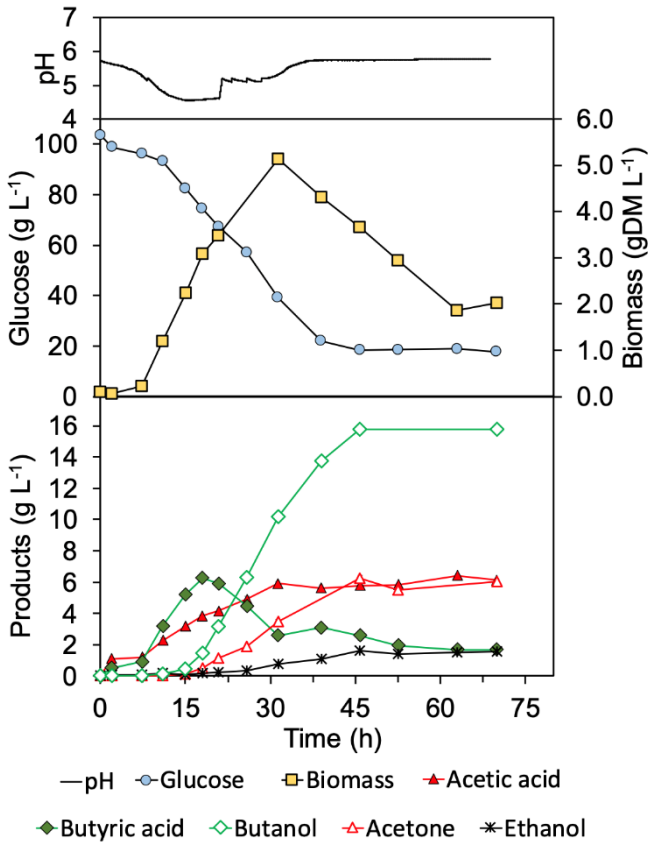


Figure 6.4. Batch fermentation on butanol production under hybrid pH control with 100 g L^{-1} of initial glucose concentration by *C. acetobutylicum.*

The maximum butyric acid of 6.25 g L^{-1} was obtained at ~18 h with a pH of 4.57. At this time-point the total undissociated acids concentration was 71.73 mM, which could have caused an acid crash. Even though the pH was increased to 5.1 at ~21 h, the fermentation was able to avoid the acid crash. Indeed, butyric acid was reassimilated during solventogenesis with a final concentration as low as 1.68 g L^{-1} (~1.2-fold increase over the reactor with pH_{min} at 5.1). In this assay 85.9 g L^{-1} of glucose (83% of total) was consumed, thus showing no improvement in carbon source uptake over the experiment with pH_{min} at 5.1. The glucose consumption rate was 2.55 g L^{-1} h^{-1}, an intermediate value between the no pH control and the pH_{min} control at 5.1. The maximum growth rate was 0.223 h^{-1} which matched the values previously obtained. The highest biomass concentration was achieved in these conditions, reaching a concentration of 3.95 g L^{-1} at ~31 h. A delay of 6-8 h in the maximum concentration was found, which was attributed to the activation of the pH control at 21 h (time point when biomass nearly peaked in previous experiments, Figure 6.3). The time-point recommended for the sudden pH step may thus be at the mid-final exponential growth phase when the minimum pH is reached. These results show that fermentation pH has a direct impact on butanol biosynthesis and glucose uptake, however, this pH strategy might be difficult carry out as it is dependent on time and acid concentration. Indeed, glucose availability could be a key factor in developing a proper pH control strategy. For example, Yang et al. (2013) carried out a time dependent (12, 24 and 36 h) pH control procedure for 50 g L^{-1} of glucose and butanol production was only improved (~2.1-fold higher) when the pH was modified at 12 h. Our results indicate that for glucose levels of ~50-70 g L^{-1}, using pH_{min} is a more robust strategy than implementing a time dependent pH control set-points.

This work has shown that the best pH control strategy depends on the initial glucose level (low, medium and high). At the low level (33 g L^{-1} of initial glucose) uncontrolled pH was more efficient, while at the medium level (66 g L^{-1} of initial glucose) using controlled pH_{min} was the best alternative. Lastly, at the high level (100 g L^{-1} of initial glucose) the hybrid pH strategy provided the highest butanol production. These promising results might be validated under more realistic conditions by checking the effect of other sugars or potential inhibitors on product yields. Future research will therefore focus on testing these pH control strategies with lignocellulosic wastes hydrolysates.

6.5. Conclusion

This study aimed to determine the most favourable pH control strategy for maximizing butanol production and glucose consumption by *C. acetobutylicum* DSM 792

within a broad range of initial glucose concentrations. For an initial glucose concentration of 33 g L^{-1}, formulating the media with an alkalinity buffer was shown to be the best alternative to enhance butanol production. For the experiments with 33 g L^{-1}, lower butanol concentration was achieved, indicating that this strain needs a higher sugar concentration to compete with other strains such as *C. beijerinckii*. Otherwise, when enough glucose is available (>66 g L^{-1}), the results indicate the superiority of controlling minimum pH as a way of increasing solvent production in ABE fermentation. When high sugar concentrations are present (100 g L^{-1}) a more sophisticated pH control strategy coupled with the acid production pattern can substantially improve the butanol titer. These results are of practical interest for establishing *ad-hoc* strategies in ABE fermentation from lignocellulosic waste, as the best pH control strategy has been shown to depend on initial sugar concentration. The experimental process proposed here can also be extended to other *Clostridium* species.

CRediT authorship contribution statement

M. Capilla: Investigation, Formal analysis, Writing - original draft, Visualization. **P. San-Valero**: Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision. **M. Izquierdo**: Conceptualization, Methodology, Visualization. **J.M. Penya-roja**: Conceptualization, Methodology, Resources. **C. Gabaldón**: Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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7. THE INFLUENCE OF SUGAR COMPOSITION AND PH REGULATION IN BATCH AND CONTINUOUS ACETONE-BUTANOL-ETHANOL FERMENTATION

In this chapter, the experimental was broadened to consider not only glucose but also xylose as carbon source, which is the second main fermentable monosaccharide of lignocellulosic substrates. For this purpose, the effect of pH strategies were evaluated using xylose and glucose:xylose mixtures as substrate. Previous buffer formulation of calcium carbonate was compared with a new buffer formulation of ammonium acetate at different proportions of glucose:xylose. Next, the best strategy was selected for the development of a continuous system to improve butanol productivity, which included the retention of the biomass with an inert matrix of plastic rings. The configuration was tested in a range of dilution factors to assess the butanol productivity and the monosaccharide consumption.

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THE INFLUENCE OF SUGAR COMPOSITION AND PH REGULATION IN BATCH AND CONTINUOUS ACETONE-BUTANOL-ETHANOL FERMENTATION

Abstract

Acetone-butanol-ethanol (ABE) fermentation is influenced by the external conditions. This work aimed to study the influence of pH regulation on monosaccharide composition in batch and continuous fermentation processes to determine butanol production and productivity. Batch fermentations with ammonium acetate or calcium carbonate combined with minimum pH control (pH≥4.8 or 5.1) were assessed with pure xylose and glucose/xylose mixtures (ratio 1:1 and 3:1). Continuous two-stage fermentation was developed using plastic rings to retain the biomass. Although batch fermentations with pure xylose performed better without active minimum pH control with both buffers, minimum pH control was necessary to metabolize xylose in the presence of glucose. Xylose uptake was favoured by the use of calcium carbonate and pH≥5.1 at a ratio of 1:1, while ammonium acetate and pH≥4.8 was the best option for a 3:1 ratio. The best butanol production and productivity values with sugar mixtures in batch reactors were 8.8 g L^{-1} and 0.61 g L^{-1} h^{-1} with ammonium acetate pH≥4.8 (ratio 3:1). The glucose/xylose ratio combined with pH regulation thus modulated xylose metabolism and solvent production in batch modes. Immobilized cells combined with operating at D=0.333 h^{-1} and pH regulation increased butanol productivity almost fourfold up to 2.4 ± 0.2 g L^{-1} h^{-1}.

Keywords: ABE fermentation; pH control; calcium carbonate; ammonium acetate; monosaccharides; continuous fermentation

7.1. Introduction

Interest in developing of biofuels has greatly increased due to the rapid depletion of fossil fuels and the rising production of greenhouse gases (Bharathiraja et al., 2017). Because of its better physicochemical properties than other biofuels such as ethanol, biobutanol is being widely investigated (Dürre, 2007). Biobutanol can be transported and delivered by the existing infrastructures, which would reduce the overall cost of implementation (Bharathiraja et al., 2017; Ibrahim et al., 2017). It can be produced by solventogenic *Clostridium* via acetone-butanol-ethanol (ABE) fermentation, which can use monosaccharides from lignocellulosic biomasses, mainly glucose and xylose, to obtain second generation biobutanol (Narisetty et al., 2022).

However, the microorganism is not efficiently capable of uptaking different sugars simultaneously due to carbon catabolite repression (CCR) (Ren et al., 2010). This phenomenon can be a problem, as it may cause sequential sugar uptake and increase residence time and overall costs. To overcome this drawback several measures have been considered, like fermentation conditions or metabolic engineering (Birgen et al., 2019). The operational conditions may change the outcome of the ABE fermentation; parameters, such as, carbon source (cellobiose, lactose or glucose) (Buendia-Kandia et al., 2018; Díez-antolínez et al., 2016; Qureshi and Blaschek, 1999), media formulation (buffer components, nitrogen source or metals) (Monot et al., 1982; Raganati et al., 2015; Valles et al., 2020) or pH (W. Jiang et al., 2014) play an important role in developing and producing bacteria.

The combination of acid overproduction and low pH can lead to acid crash due to an undissociated acid concentration boost. These undissociated acid species can permeate through the cell, preventing sugar uptake and solvent production (Maddox et al., 2000). To avoid this, pH can be controlled by buffering compounds like calcium carbonate (Yang et al., 2013) or ammonium acetate (Ezeji et al., 2004). Focused chemical control strategies can also be used to regulate the pH value with *C. acetobutylicum*. Batch fermentations are the easiest and most conventional configurations for ABE fermentation (Bharathiraja et al., 2017). For example, batch mode with different levels of initial glucose (33, 66 and 100 g L^{-1}) (Capilla et al., 2021); with a two-stage pH control strategy (Guo et al., 2012) or with lignin-derived inhibitors from lignocellulosic pretreatment (Luo et al., 2019) have been tested.

In contrast, although continuous fermentation requires close process control, it allows increased productivity and sophisticated capabilities in the fermentation process

(Vees et al., 2020). The recent implementation of cell immobilization, which is under investigation mainly using glucose systems (Dolejš et al., 2014; Liu et al., 2014), has certain advantages, such as prevention of microorganism washout, uncoupling the dilution rate and the maximum growth rate or minimizing the propagation cost. However, there are other disadvantages such as clogging, or mass-transfer limitations (Vees et al., 2020).

In the present work, a study was made of the influence of pH regulation on monosaccharide composition to evaluate butanol production and productivity in ABE fermentation. To assess the shared effect of buffer formulation and active pH control with pure xylose and glucose/xylose ratios (1:1 and 3:1), a novel pH-controlled continuous cell retention ABE fermentation was carried out in the optimal conditions of batch fermentations to further increase butanol productivity.

7.2. Materials and Methods

7.2.1. Microorganism and medium fermentation

C. acetobutylicum DSM 792 was acquired from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and maintained in 20% glycerol at −80 °C. For seed inoculum, the bacteria were statically cultured at 37° in 19 g L^{-1} of Reinforced Clostridial Medium (RCM) supplemented with 10 g L^{-1} of glucose. The sterilized fermentation medium contained (g L^{-1}): sugars, 60 (glucose, xylose or sugar mixture); yeast extract, 5; KH_2PO_4, 0.5; K_2HPO_4, 0.5; resazurin sodium salt, 0.001; antifoam 204, 0.01%; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 7H_2O$, 0.01; and $FeSO_4 \cdot 7H_2O$, 0.05. Two buffers were studied in batch fermentations: ammonium acetate and calcium carbonate bases. The buffers tested were ammonium acetate, 2.2 g L^{-1} or calcium carbonate, 5 g L^{-1} combined with NH_4Cl, 2 g L^{-1}. The chemicals were purchased from VWR, except for $CaCO_3$ (Merk, Darmstadt, Germany), antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA) and yeast extract (Alfa Aesar, Haverhill, MA, USA).

7.2.2. Experimental set-up

7.2.2.1. Batch fermentations

Batch fermentations were carried out using 0.8 L on effective volume in a 1.1 L total volume reactor (Figure 7.1A). Anaerobic conditions were accomplished before inoculation by insufflating N_2 for 30 minutes. The assays were carried out with an inoculum size of 5% at 120 rpm and 37ºC. The fermentations were monitored by a Tris-

compatible flat pH sensor connected to LoggerPro software (Vernier, Beaverton, OR, USA). A feedback control strategy on/off was used to avoid that the pH decreased below a set point value (4.8 or 5.1 depending of the experiment). NaOH (3 M) was automatically added when the pH reached the lower limit (dead band 0.1) (Figure 7.1C). The upper limit was not controlled, allowing the recovery of the pH when possible. Samples were collected at specific time-points to analyse cell growth and metabolites.

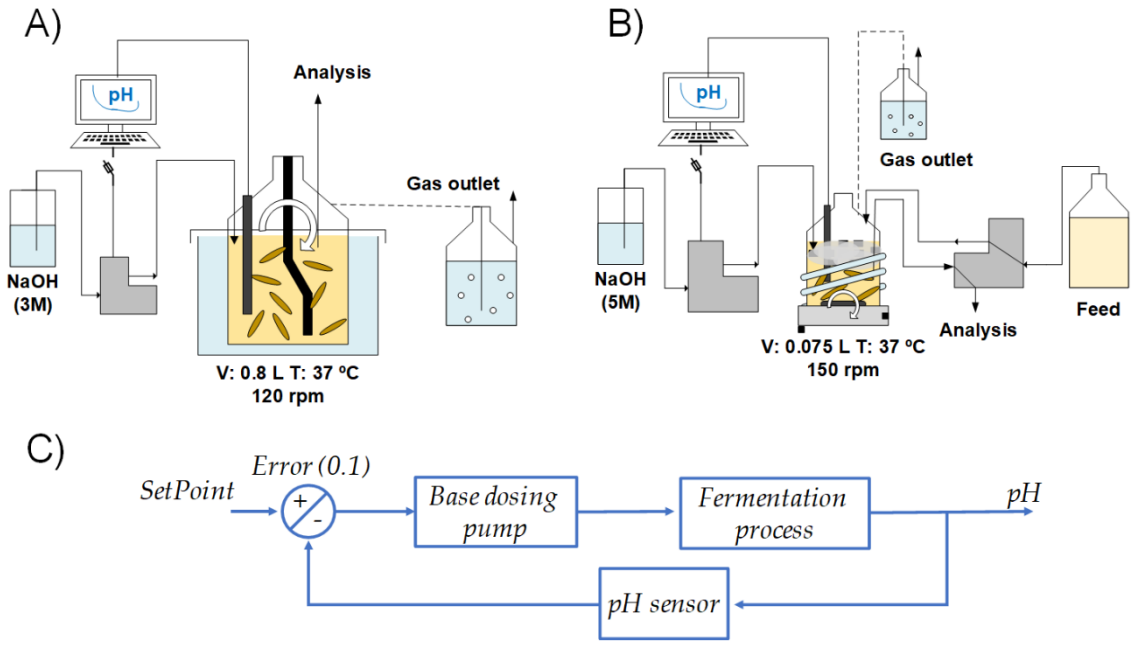

Figure 7.1. Fermentations set-up with *C. acetobutylicum* DSM 792. A) Batch fermentations, B) continuous fermentation and C) block diagram for the pH.

The experimental plan is summarized in Table 7.1. The use of xylose was tested as it is the second main monosaccharide in lignocellulosic hydrolysates. The reduction of xylose concentration concomitant with incrementing the glucose was used to evaluate the effect of monosaccharide composition which would mimic a spectrum of lignocellulosic hydrolysates concentrations. In that sense, three different glucose/xylose mass ratios (0:1, 1:1, 3:1, final monosaccharide concentration: 60 g L^{-1}) were tested in batch mode. For every sugar composition, the effect of buffer coupled or not with a strategy of limiting the minimum pH was evaluated. Both buffers were selected as previously demonstrated good performance in ABE fermentation (Capilla et al., 2021; Monot et al., 1982). The set-points were chosen from a previous work with glucose (Capilla et al., 2021).

Table 7.1. Experimental plan with batch mode. The value of pH control indicates the minimum pH reached.

Glucose/xylose ratio	Ammonium acetate (2.2 g L^{-1})		Calcium carbonate (5 g L^{-1})	
	Blank	pH control	Blank	pH control
0:1	Yes	4.8	Yes	5.1
1:1	Yes	4.8	Yes	5.1
3:1	Yes	4.8	Yes	4.8 and 5.1

7.2.2.2. Continuous fermentation

A stirred continuous fermentation was carried out using 0.075 L of effective medium in a 0.25 L reactor (Figure 7.1B). The medium was prepared as described previously with ammonium acetate as buffer. The configuration used a multichannel peristaltic pump (Reglo ICC, Ismatec, Wertheim, Germany) at the desired equal in-and-out flow rate. Plastic rings were used as packing material at 7.5% w/v. Before inoculation, anaerobic conditions were accomplished by insufflating N_2 for 10 minutes. The fermentation was carried out with an inoculum size of 5% at 150 rpm and 37ºC. The temperature was maintained by a water heating coil while stirring with a magnetic stirrer. An identical pH monitoring protocol was used as in the batch fermentations based on the automatic limitation of minimum pH. A two-stage pH reactor was developed by chemically controlling the fermentation at two different minimum set-points (6.0 and 4.8) using NaOH (5M). The summary conditions of the experiment are shown in Table 7.2. Glucose was used as carbon source and pH≥6.0 to favour acidogenesis conditions in the biomass growth phase, performing the batch mode in the first 30 h, after which the continuous mode started (dilution rate, D= 0.042 h^{-1}). During the acidogenesis phase, the dilution rate was doubled every ~48 h up to a D= 0.166 h^{-1} to improve productivity. After that, at 144 h, the pH was allowed to drop to pH≥4.8 to favour the solventogenesis phase.

Table 7.2. Summary conditions of the continuous fermentation

Time (h)	Minimum pH set point	Substrate	Concentration (g L^{-1})	D (h^{-1})
0-30	6.0	Glucose	60	Batch
30-72	6.0	Glucose	60	0.042
72-121	6.0	Glucose	60	0.083
121-144	6.0	Glucose	60	0.167
144-192	4.8	Glucose	60	0.167
192-216	4.8	Glucose	60	0.333
216-288	4.8	Glucose/Xylose	40:20	0.333

7.2.3. Analytical methods

Suspension cell density (gDW L^{-1}) was quantified at 600 nm (OD_{600}) using a UV–Vis spectrophotometer (SpectroFlex 6600, WTW, Weilheim in Oberbayern, Germany) as gDW L^{-1} = 0.2941·OD_{600} + 0.0331 (R^2 = 0.9908). Samples were centrifuged (10,000 rpm, 5 min) and 0.22 µm filtered. Metabolites of interest were quantified using liquid chromatography (Agilent 1100 Series HPLC system, Agilent Technologies, Santa Clara, CA, USA) coupled with an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 50 °C. A refractive index detector (RID) at 35 °C was utilized to detect monosaccharides, ethanol and butanol, while a diode array detector (DAD) was used at 210 nm to detect butyric and acetic acids and at 280 nm to detect acetone. Five mM of sulfuric acid was used as mobile phase at 0.6 mL min^{-1} for 40 min. The Henderson–Hasselbalch formula was used to quantify the undissociated acids (pK_aacetic: 4.76; pK_abutyric: 4.82). The maximum production rates in batch were estimated at the exponential phase of each compound by Equation 7.1:

$$r = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \quad (7.1)$$

Where r is the production rate (g L^{-1} h^{-1}). S_{t1} and S_{t2} are the species concentrations at the initial and final point of the exponential phase (g L^{-1}), t_2 and t_1 are the starting and ending times of the exponential phase (h). For continuous fermentations, productivity was estimated as follows (Equation 7.2):

$$P_s = S \cdot D \quad (7.2)$$

Where, S is the average species production (g L^{-1}) and D, is the dilution rate (h^{-1}).

7.3. Results and discussion

7.3.1. Batch fermentation

Two buffer media (calcium carbonate/ammonium acetate) and their combination with an active pH control were compared for 60 g L^{-1} of xylose (Figure 7.2). The evolution of the pH is shown in Figure S7.1A. The growth of the bacteria using xylose was characterized by an initial lag-phase of 12-24 h, after which the exponential growth started (Figure 7.2G). The use of calcium carbonate increased biomass concentration 1.4-fold over the ammonium acetate in spontaneous fermentation, indicating that calcium carbonate enhanced biomass production. The combination of buffer and pH control clearly changed the biomass pattern; the maximum biomasses increased from 4.2 to 7.2

g L^{-1} for the acetate reactors (blank and pH≥4.8) and from 5.8 to 7.9 g L^{-1} for the carbonate reactor (blank and pH≥5.1). This increase represented about ~1.7 and 1.4 times with ammonium acetate and calcium carbonate, respectively. Similar xylose consumptions were obtained whatever the buffer used and the pH set-point employed, with no complete depletion of the monosaccharide (Figure 7.2A). This was because a butanol concentration of ~4.50 g L^{-1} would reduce initial xylose uptake rate by 50%. A titer of ~8.0-8.5 g L^{-1} has been determined to be growth inhibitory for *C. acetobutylicum* on xylose as carbon source (Ounine et al., 1985; Raganati et al., 2015). The impact of the buffer can be seen in the butyric acid concentration (Figure 7.2E). Calcium carbonate almost doubled (6.6 over 3.4 g L^{-1}) the maximum concentration of the acid against ammonium acetate with no pH control. Under the conditions tested with xylose as sole carbon source, both buffers performed better with spontaneous fermentation without pH control. Butanol (ABE) productions were 8.0 (13.1) and 5.3 (8.1) g L^{-1} for the acetate reactor (blank and pH≥4.8) while 7.8 (11.8) and 5.8 (8.1) g L^{-1} for the carbonate reactor (blank and pH≥5.1). These results show that the minimum pH control was detrimental with either ammonium acetate or calcium carbonate buffers under these conditions. Previous results showed that when 60 g L^{-1} glucose was used as a sole carbon source, the strategy of limiting the minimum pH at 5.1 enhanced butanol (ABE) production from 7.47 (10.50) g L^{-1} to 11.22 (16.12) g L^{-1} (Capilla et al., 2021). As xylose uptake behaved differently to glucose, which is the major monosaccharide of lignocellulosic residues, the effect pH regulation (active pH control and/or buffer media) with its mixtures should be investigated.

Based on the xylose results, fermentation with mixtures of glucose/xylose (final monosaccharide concentration: 60 g L^{-1}) were developed for two sugar compositions. A 1:1 sugar ratio was first used (Figure 7.3). The pH evolution can be found in Figure S7.1B. Biomass concentrations were about 60% lower than those obtained with pure xylose, resulting in values of 2.5–3.2 g L^{-1}, attributed to the lower biomass yield linked to glucose consumption. Capilla et al. (2021) estimated a maximum biomass concentration of about 3 g L^{-1} with 33 g L^{-1} of pure glucose. The case of using calcium carbonate and pH control strategy had a notably different behaviour. This exhibited diauxic growth linked with the xylose uptake from hour 48 onwards after 24 h of pure glucose depletion (Figure 7.3A and 7.3B). Butyric acid production linked to the consumption of both substrates, also occurred using calcium carbonate and the pH minimum of 5.1, first at a rate of 0.52 g L^{-1} h^{-1} and then 0.18 g L^{-1} h^{-1} (Figure 7.3F).

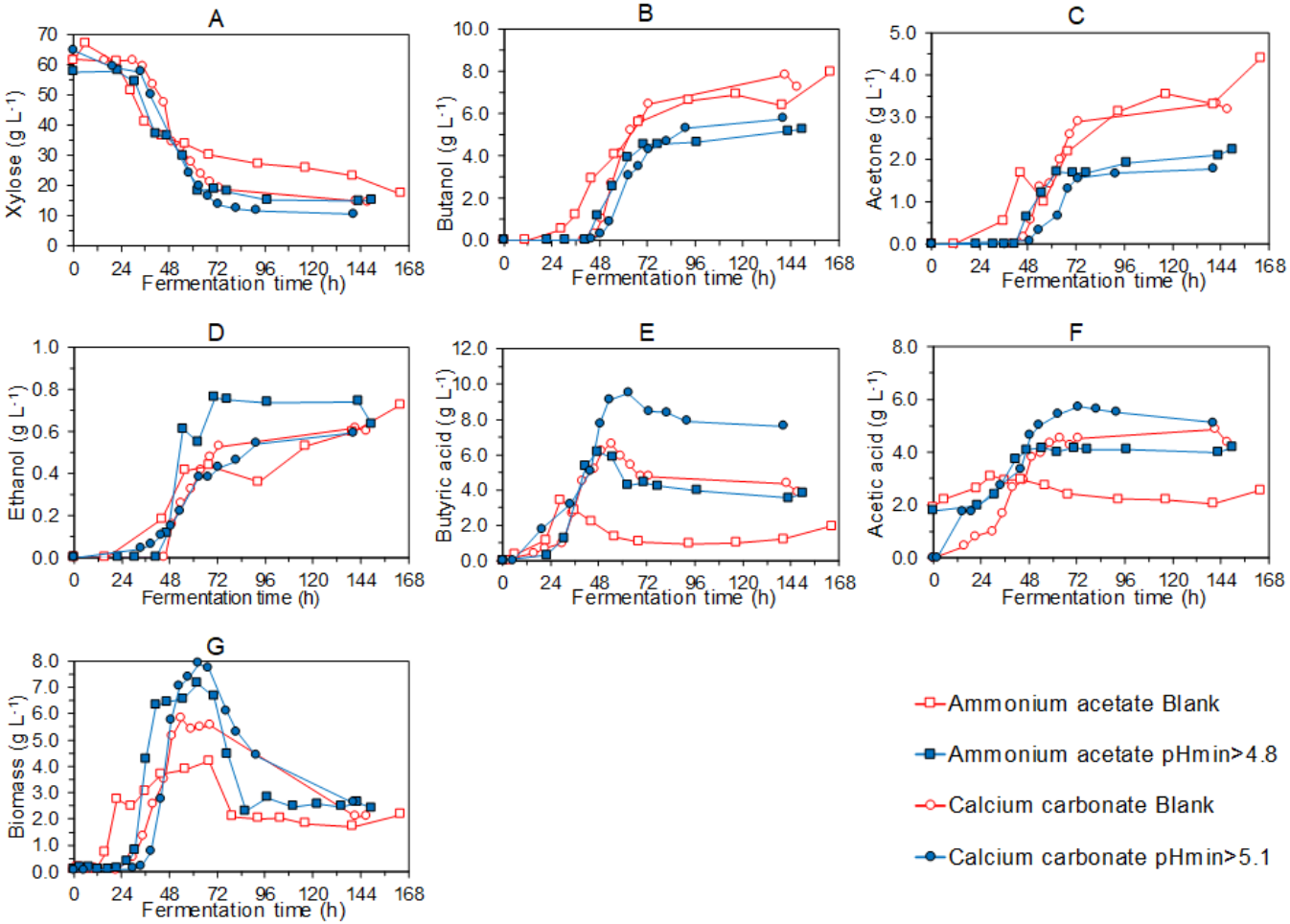

Figure 7.2. Fermentation profiles of (A) xylose, (B) butanol, (C) acetone, (D) ethanol, (E) butyric acid, (F) acetic acid and (G) biomass in g L^{-1} with *C. acetobutylicum* with pure xylose in batch mode.

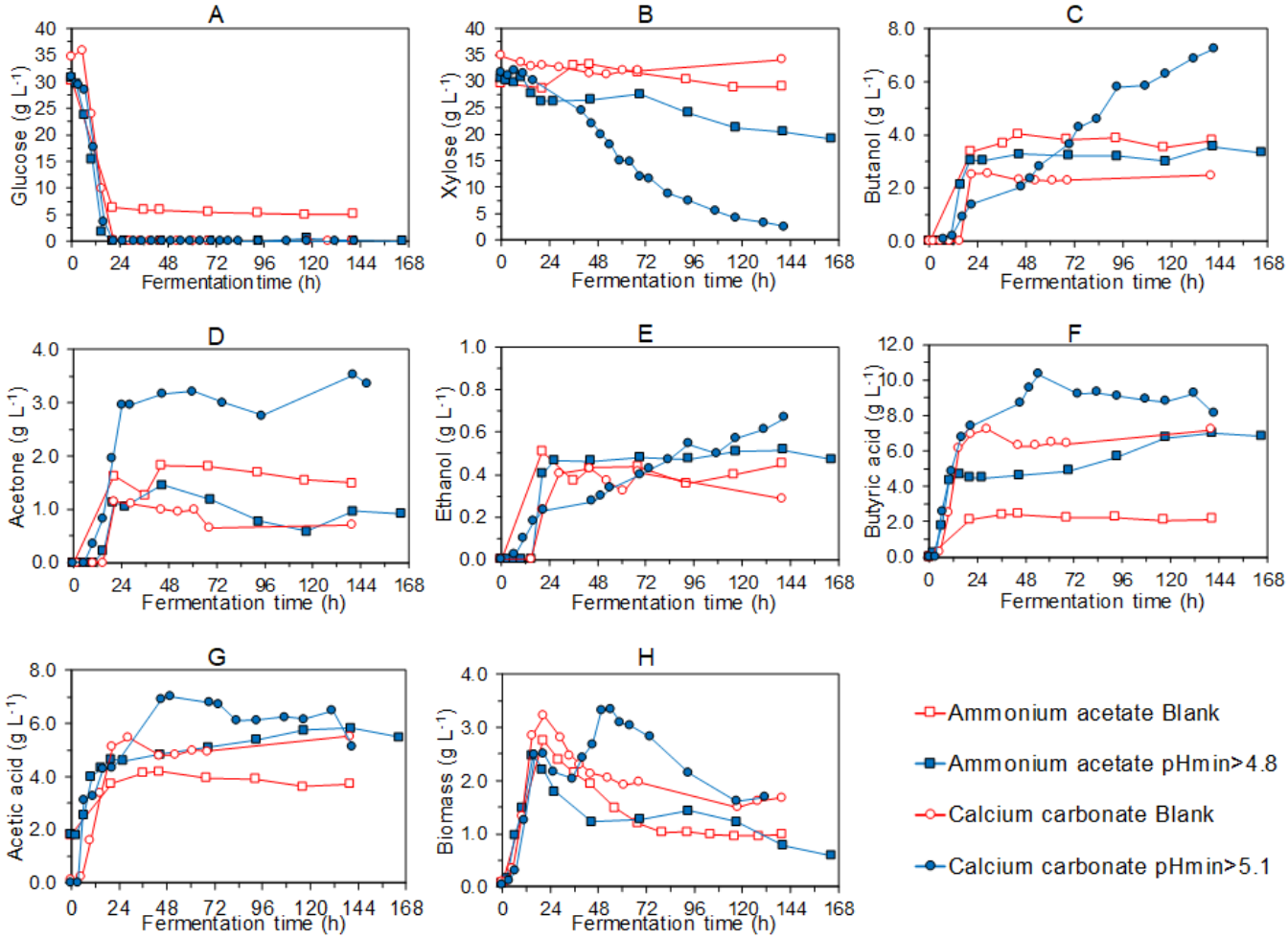

Figure 7.3**.** Fermentation profiles of (A) glucose, (B) xylose (C) butanol, (D) acetone, (E) ethanol, (F) butyric acid, (G) acetic acid and (H) biomass in g L^{-1} with *C. acetobutylicum* with 1:1 ratio of glucose:xylose in batch mode.

This second butyric production resulted in a peak of 10.3 g L^{-1} of butyric acid, similar to the maximum concentration achieved with pure xylose (Figure 7.2E). As can be seen, this is the only operational condition in which xylose uptake was concomitant to butanol production (7.2 g L^{-1}) (Figure 7.3B). The beneficial effect of calcium carbonate as buffer regulator was also found by El Kanouni et al. (1998). Otherwise, a pH of 4.8 does not produce good xylose uptake when ammonium acetate is the buffer, so that the buffer regulation itself has an impact on xylose uptake for a specific mixture of glucose and xylose. This highlights the importance of a tailor-made strategy not only for pH conditions but also the type of buffer. These results show the potential of *C. acetobutylicum* for biobutanol production when sugar mixtures of glucose and xylose are employed, which could be the case of lignocellulosic substrates, among others.

To mimic the conditions of lignocellulosic hydrolysates, a glucose/xylose ratio of 3:1 (final monosaccharide concentration: 60 g L^{-1}) was also tested and the same conditions as with previous experiments were evaluated. Calcium carbonate was tested at two pH of 4.8 and 5.1 to elucidate the impact of the control of different minimum pH. The pH profiles are shown in Figure S7.1C. A similar biomass production was obtained by comparing it with the 1:1 glucose/xylose mixture at a maximum concentration of about 2.7–2.9 g L^{-1} for the non-pH-controlled reactors and pH-controlled reactor with calcium carbonate. A higher biomass concentration was achieved with ammonium acetate and pH control (Figure 7.4). Reducing the xylose level and increasing glucose, no diauxic growth occurred in the pH-controlled reactor buffered with calcium carbonate at either pH 5.1 or 4.8. Glucose consumption stopped either by end of the fermentation due to acid crash (blank) or glucose exhaustion (pH≥4.8-5.1) with both buffers (Figure 7.4A). Unlike ratio 1:1, calcium carbonate fermentation with either active pH control (pH≥4.8-5.1) did not show any xylose uptake after glucose depletion with a sugar ratio of 3:1. As the failure of xylose metabolisms with calcium carbonate as buffer reagent could be attributed to the low xylose concentration on the media (15 g L^{-1}), it seems that a minimum concentration of xylose is required to modulate the pathway to xylose by *C. acetobutylicum*.

In contrast, the use of ammonium acetate instead of calcium carbonate promoted xylose depletion, although the xylose uptake started later (72 h and 48 h after glucose depletion) than in the previous experiment, which also shows that low xylose concentrations complicate the metabolic shift from glucose to xylose consumption. Indeed, there was no biomass growth associated with xylose consumption, as when using 30 g L^{-1} of xylose. The monosaccharide would therefore be used for cell

maintenance rather than biomass production. The lower effectiveness of pH-controlling strategies on xylose uptake at higher ratios of glucose/xylose was also found by Jiang et al. (2014). For the experiments without noticeable xylose uptake, butanol (ABE) production ranged from 3.6–4.9 (5.2-8.2) g L^{-1}, which is in agreement with the higher glucose from a prior experiment (2.6-4 g L^{-1} butanol production with 30 g L^{-1} of glucose). The ammonium acetate with pH≥4.8 experiment successfully consumed nearly all the xylose, and maximum butanol (ABE) production was 8.8 (13.2) g L^{-1}. This concentration was very similar regardless of sugar composition when 60 g L^{-1} of either xylose or xylose and glucose were used, although the type of buffer regulation and pH control parameters were different for each case. This was related to the butanol concentration, as 8-8.5 g L^{-1} has been shown to be inhibitory when *C. acetobutylicum* is grown with xylose (Ounine et al., 1985; Raganati et al., 2015). The best condition at ratio 3:1 was pH control at 4.8 and ammonium acetate as buffer reagent. In these conditions, maximum butanol (ABE) productivity was 0.61 g L^{-1} h^{-1} (0.89 g L^{-1} h^{-1}). Fermentation in continuous mode was also tested to improve butanol productivity.

7.3.2. Continuous fermentation

The pH regulation strategy was evaluated in a continuous fermentation filled with plastic rings to operate at high D values while preventing biomass washout. To promote biofilm growth, the pH was first kept at 6.0, after which pH was shifted to the minimum of 4.8 to promote solvent production. During the biofilm formation stage, it was fed 60 g L^{-1} of glucose with a pH≥6.0 (acidogenic optimum pH) at a dilution factor of 0.042 h^{-1} (Figure 7.5). The biomass was seen to grow on biofilm interlacing with the plastic rings. After 72 h (2 residence time (θ) from start of continuous fermentation), the biomass concentration leaked less than 3 g L^{-1}, indicating successful biomass retention. At this time, the dilution factor was doubled to 0.083 h^{-1}, with a transitory biomass leak (96 h). Thereafter, the suspended biomass remained stable for the rest of the experiment at 3.9 ± 0.9 g L^{-1}, indicating no washout on the duplication of the dilution factor until the end of the experiment (maximum dilution rate 0.333 h^{-1}). This configuration allowed a fast start-up and high biomass retention. At 120 h, the dilution factor was again doubled to 0.167 h^{-1}. As solventogenesis was inhibited in this pH, the conditions were very low solvent production and high acid concentrations. At 144 h (D= 0.167 h^{-1}), pH was allowed to drop to the solventogenesis set point (pH≥4.8). In less than 24 h, the acids were consumed while butanol (ABE) was produced. In steady state conditions, a butanol (ABE) concentration of 9.0 ± 2.0 (10.1 ± 1.1) g L^{-1} (productivity: 1.5 ± 0.3 (1.7 ± 0.2) g L^{-1} h^{-1}) was achieved, indicating proper transition to solventogenesis.

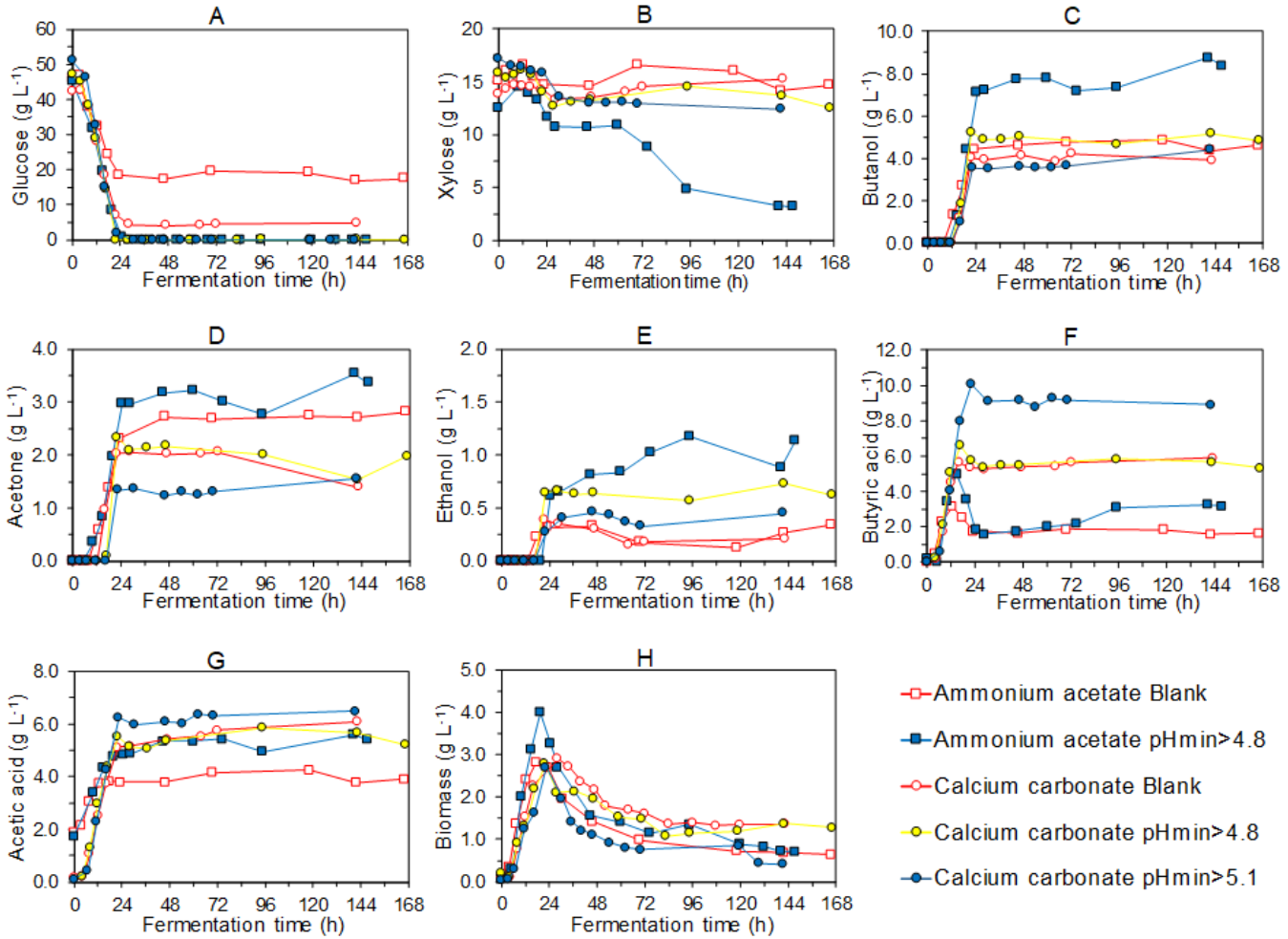

Figure 7.4. Fermentation profiles of (A) glucose, (B) xylose (C) butanol, (D) acetone, (E) ethanol, (F) butyric acid, (G) acetic acid and (H) biomass in g L^{-1} with *C. acetobutylicum* with 3:1 glucose:xylose ratio in batch mode.

The butanol (ABE) concentration achieved here was close to the maximum reported in batch reactors with the same glucose concentration and similar pH modulation strategy (Capilla et al., 2021). At 192 h, the dilution factor was again doubled to 0.333 h^{-1}. After 8 θ, steady state butanol values (ABE) were 6.8 (11.1) g L^{-1} with a productivity of 2.3 (3.7) g L^{-1} h^{-1}. At 216 h, glucose input was thus changed to glucose/xylose mixture (2:1 ratio) to test the effect of varying the monosaccharide concentration, after which the butanol concentration was kept stable for at least 24 θ, with an average butanol (ABE) concentration of 7.1 ± 0.6 (11.2 ± 1.0) g L^{-1}. This concentration led to a butanol (ABE) productivity of 2.4 ± 0.2 (3.7 ± 0.3) g L^{-1} h^{-1} with a mixture 2:1 glucose/xylose. Additionally, the mass balance analysis is presented in Table 7.3. As expected, the distribution of the products manifested opposite trends depending on the pH set point. Butyric acid was the main product at pH≥6.0 while butanol was the major product at pH≥4.8. Moreover, the unaccounted yield would be associated to biofilm formation and CO_2 production. In this sense, previous authors reported that in ABE fermentation, the CO_2 could be up to a third of the metabolites produced (Darkwah et al., 2018).

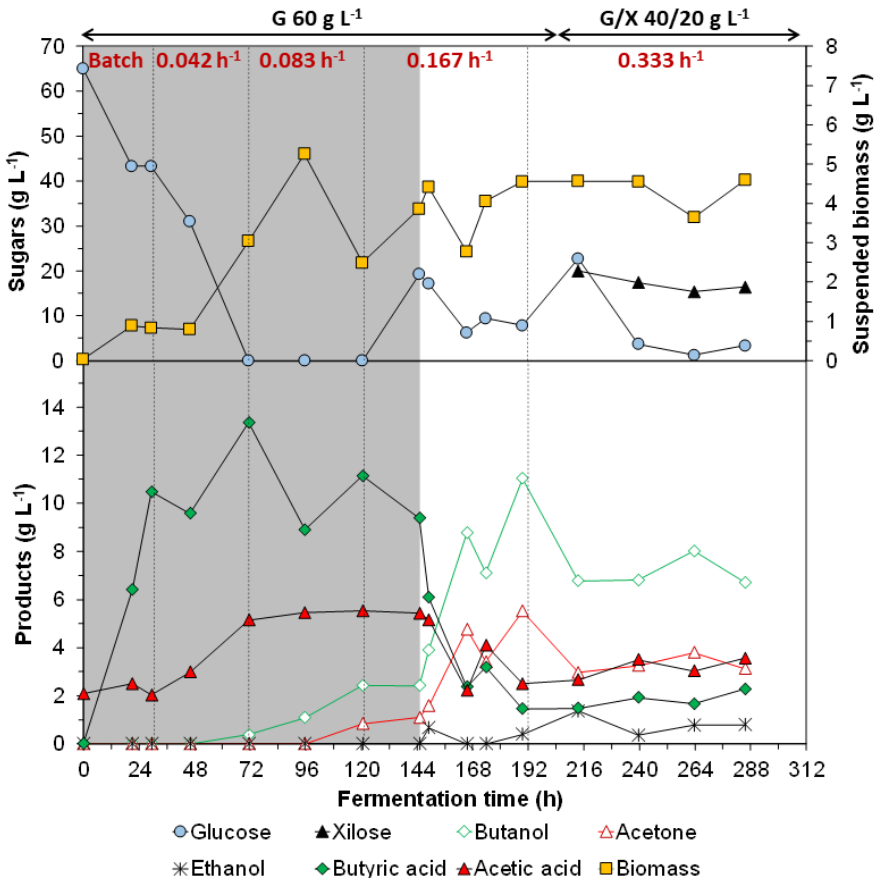

Figure 7.5. Fermentation profile of *C. acetobutylicum* with plastic rings at different dilution rates (h^{-1}) and monosaccharide feed. Grey area shows acidogenic phase (pH≥6.0) while unshaded area is solventogenic phase (pH≥4.8). G denotes glucose and X xylose.

The productivity obtained with a 2:1 glucose/xylose mixture (D= 0.333 h^{-1}) is almost four times more than that obtained in the batch reactor at a ratio of 3:1 with ammonium acetate and pH≥4.8 (butanol (ABE) productivity of 0.61 (0.89) g L^{-1} h^{-1}). Using sugarcane bagasse for biomass immobilization, solvent productivities in the range of 1.8 - 2.1 g L^{-1} h^{-1} at D ranging 0.3-0.6 h^{-1} have been obtained (Bankar et al., 2012). In continuous operation mode at dilution factors ≥ 0.167 h^{-1}, glucose uptake was about 85% when 60 g L^{-1} was fed, while full consumption was at 40 g L^{-1} and xylose uptake was only 18 ± 8%. The lower xylose uptake than the batch reactor (74%) can be related to the continuous availability of glucose, which would mostly inhibit extending the shift of the metabolism to xylose. This shows the feasibility of developing an efficient continuous reactor configuration avoiding biomass washout and obtaining high stable productivities. However, further investigation is required to enhance the use of xylose in continuous operations.

7.4. Conclusions

The glucose/xylose ratio has a direct impact on the selection of the proper pH modulation strategy (pH control and/or buffer composition) in ABE fermentation using *C. acetobutylicum* DSM 792. Tailor-made pH modulation strategies should be developed for specific glucose/xylose mixtures to use the substrate. For a mixture of pure xylose (0:1), both buffers tested (calcium carbonate/ammonium acetate) led to similar results in terms of butanol production. However, minimum pH control worsened the solvent production. In the case of 1:1 glucose/xylose mixtures, calcium carbonate as buffer reagent combined with the strategy of limiting minimum pH to 5.1 gave the best results, achieving nearly full consumption of xylose while producing close to 8 g L^{-1} of butanol. When the glucose/xylose ratio was raised to 3:1, the buffer ammonium acetate with pH≥4.8 performed better, achieving a butanol concentration of 8.8 g L^{-1} and almost complete xylose uptake. The continuous reactor configuration filled with plastic rings for biomass retention successfully increased butanol (ABE) productivity while preventing biomass washout. This configuration led to a biobutanol productivity of 2.4 ± 0.2 g L^{-1} h^{-1} at D= 0.333 h^{-1} with a sugar mixture of 2:1, which is almost four times greater than that obtained in batch operations. However, in this configuration xylose consumption worsened to 18 ± 8%. Further studies should thus be carried out to improve substrate use in continuous mode.

Table 7.3. Mass balance analysis for the continuous experiment.

Time (h)	Substrate (g L^{-1})	pH	D (h^{-1})	g-C g-C of Monosaccharide Consumed^{-1}							
				Acetic Acid	Butyric Acid	Butanol	Acetone	Ethanol	Suspended Biomass	Yield	Unaccounted
0-30	Glucose (60)	6.0	Batch	0.00	0.66	0.00	0.00	0.00	0.05	0.70	0.30
30-72	Glucose (60)	6.0	0.042	0.14	0.39	0.01	0.00	0.00	0.05	0.59	0.41
72-121	Glucose (60)	6.0	0.083	0.09	0.21	0.06	0.02	0.00	0.09	0.47	0.53
121-144	Glucose (60)	6.0	0.167	0.13	0.30	0.10	0.04	0.00	0.13	0.70	0.30
144-192	Glucose (60)	4.8	0.167	0.07	0.05	0.28	0.13	0.01	0.11	0.65	0.35
192-216	Glucose (60)	4.8	0.333	0.07	0.05	0.27	0.11	0.05	0.16	0.72	0.28
216-288	Glucose/Xylose (40:20)	4.8	0.333	0.08	0.07	0.28	0.13	0.02	0.14	0.72	0.28

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8050226/s1, Figure S1, The pH profiles of *C. acetobutylicum* under different carbon sources (A) pure xylose, (B) ratio 1:1 (C) ratio 3:1 in batch mode.

Author Contributions: Conceptualization, M.C., P.S.-V., F.J.Á.-H. and C.G.; methodology, P.S.-V., F.J.Á.-H., C.G., A.V., M.C. and C.S.; formal analysis, M.C., C.S. and P.S.-V.; investigation, M.C., C.S. and A.V.; resources, F.J.Á.-H. and C.G.; data curation, M.C., C.S. and P.S.-V.; writing—original draft preparation, M.C.; writing—review and editing, P.S.-V. and C.G.; visualization, M.C., C.S., P.S.-V. and C.G.; supervision, P.S.-V. and C.G.; project administration, F.J.Á.-H. and C.G.; funding acquisition, F.J.Á.-H. and C.G. All authors have read and agreed to the published version of the manuscript.

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Appendix A. Supplementary data

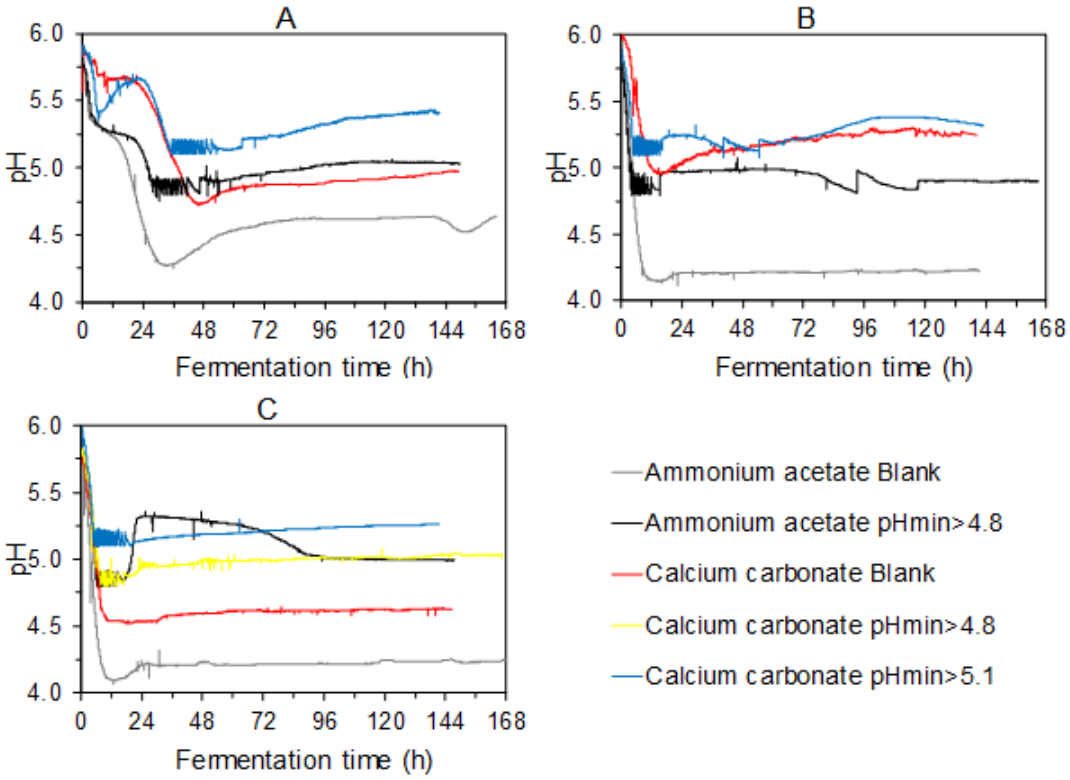

Figure S7.1. The pH profiles of *C. acetobutylicum* under different carbon sources (A) pure xylose, (B) ratio 1:1 (C) ratio 3:1 in batch mode.

8. SOLVENT PRODUCTION FROM RICE STRAW BY A CO-CULTURE OF *CLOSTRIDIUM ACETOBUTYLICUM* AND *SACCHAROMYCES CEREVISIAE*: EFFECT OF PH CONTROL

This chapter deepens on the development of strategies to further improve the exploitation of rice straw hydrolysate with C. acetobutylicum. In this chapter, the study of pH was tested not only with synthetic media but also with real rice straw hydrolysate. One of the main drawbacks observed in previous chapters is the limited xylose consumption at the sugar proportions encountered on the rice straw hydrolysate. To overcome this issue, next step on the research consisted of testing the bacteria C. acetobutylicum with the yeast S. cerevisiae in a co-culture strategy. After optimization of the inoculation and operation protocol, the influence of pH control strategy on monosaccharide uptake and solvent production was assessed with the rice straw hydrolysate.

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SOLVENT PRODUCTION FROM RICE STRAW BY A CO-CULTURE OF *CLOSTRIDIUM ACETOBUTYLICUM* AND *SACCHAROMYCES CEREVISIAE*: EFFECT OF PH CONTROL

Abstract

One of the challenges in biofuel production from lignocellulosic wastes is to improve its conversion to solvents, therefore new strategies to enhance xylose uptake are required due to be the secondary abundant sugar. In this context, a novel fermentation strategy integrating a co-culture of *Clostridium acetobutylicum* and *Saccharomyces cerevisiae* with pH control was developed. Initially, two different buffers, ammonium acetate and calcium carbonate, were tested under pH_{min}>4.8 by fermenting 60 g L^{-1} of glucose with the *C. acetobutylicum* monoculture. Ammonium acetate was selected for fermenting media as butanol production was increased from 9.8 to 10.9 g L^{-1} over the calcium carbonate test. Comparing with the spontaneous acetone-butanol-ethanol (ABE) fermentation with *C. acetobutylicum* when no xylose consumption was observed, xylose consumption was efficiently increased by controlling pH_{min}>4.8. The xylose consumption was >47% either by using a 45:15 g L^{-1} glucose:xylose mixture or with rice straw (RS) hydrolysate. *Clostridium* monoculture using RS hydrolysate and pH_{min}>4.8 produced a butanol (ABE) concentration of 6.5 (9.5) g L^{-1}. While it increased to 7.0 (13.1) g L^{-1} when the co-culture with *S. cerevisiae* was used using same pH regulation strategy mainly due to ethanol increase up to 2.7 g L^{-1}. Moreover, the xylose uptake doubled to 94% due to amino-acid secretion by yeast. Overall, this combined strategy was a very effective method for promoting sugar consumption and ABE solvent production from lignocellulosic waste.

Keywords: ABE fermentation; Ethanol fermentation; Lignocellulosic waste, pH control; Xylose

8.1. Introduction

The world is moving towards a more sustainable economy based on the use of renewable sources urged, among other factors, by the climate change awareness and the necessity of limiting the greenhouse gases emission (IEA-Bioenergy, 2020). Butanol is a highly appreciated biofuel due to its physicochemical properties and would not require extensive investments as it can be delivered via current infrastructure (Bharathiraja et al., 2017; Dürre, 2007; Ibrahim et al., 2017). Biobutanol can be produced via Acetone-Butanol-Ethanol (ABE) fermentation by solventogenic *Clostridia*. However, ABE fermentation has downsides as low concentrations or substrate costs. First-generation biobutanol was produced by sugar- or starch-based feedstock (like sugarcane or corn) with drawbacks as high cost of raw materials and competition in the food supply. Thus, the lignocellulosic biomasses from agro-food activities have grown in interest due to low cost and wide availability (Xue et al., 2013). Lignocellulosic biomasses, such as rice straw (RS), require a pretreatment prior to sugar release from cellulose and hemicellulose after enzymatic hydrolysis. Among the pretreatments, alkalis are capable of swelling the cellulose structure and remove acetyl groups, lignin and uronic acid substitutions, hence increasing enzyme accessibility to the polysaccharides (Haghighi Mood et al., 2013). From a compendium of 77 hydrolysed lignocellulosic feedstock, Birgen et al. (2019) obtained a median value of 23.6 and 10.8 g L^{-1} of glucose and xylose (glucose:xylose ratio of ~2.2:1); thus, xylose is the secondary monosaccharide in hydrolysates composition. However, the carbon catabolite repression (CCR) of glucose over xylose hinders the overall efficiency of ABE fermentation (Ren et al., 2010), being one of the drawbacks when using lignocellulosic biomass.

Regarding solventogenic *Clostridium* species, it was reported that *Clostridium beijerinckii* is capable of uptake xylose efficiently in presence of glucose (Zhang et al., 2016), which could be due to the presence a big gene cluster of D-xylose pathway genes found in some strains such as *C. beijerinckii* NCIMB 8052 (Xiao et al., 2012). In the case of *Clostridium acetobutylicum*, previous studies reported that the xylose catabolic routes, pentose phosphate (Gu et al., 2009) or the phosphoketolase pathway, are influenced by the level of xylose present in the media (Liu et al., 2012). Thus, more efforts should be made to elucidate strategies to better control the xylose uptake. This is especially important in the case of glucose:xylose mixtures to avoid CCR, among the singular characteristics which makes the study of *C. acetobutylicum* of interest highlights its capability to form a denser biofilm due to cell to cell communication (Liu et al., 2018),

which could led to the increase the overall productivity by promoting cell immobilization (Zhang et al., 2021).

In addition, the media pH has been shown to play an important role on the fermentation profile of *C. acetobutylicum*. For example, controlling the minimum pH on batch fermentation boosted glucose consumption and butanol production by alleviating acid crash, with an increment from 7.47 to 11.22 g L^{-1} butanol when comparing with no pH control (Capilla et al., 2021). Regulation of pH was also successfully implemented in continuous reactors with *C. acetobutylicum* using lignocellulosic substrates (Survase et al., 2019). Moreover, pH-controlled continuous fermentations have been also shown effective with xylose (Procentese et al., 2015b, 2014). Another strategy that has been shown efficient to improve sugar uptake and solvent production by *C. acetobutylicum* was formulation the media with $CaCO_3$ producing 9.65 g L^{-1} of butanol from a glucose:xylose mixture (30:30 g L^{-1}) with 82% of sugar consumption (Raganati et al., 2015). Indeed, $CaCO_3$ was demonstrated to reduce the residual xylose with glucose:xylose mixtures regardless of the monosaccharide ratio in ABE fermentation (El Kanouni et al., 1998). W. Jiang et al. (2014) also demonstrated that pH control could increment xylose uptake despite their sugar ratio. Best results were achieved at the lower glucose:xylose ratio tested (1.5:1); consumption of xylose was enhanced from 11.6% without pH control to 66.1% by controlling pH after the acidogenic phase. Therefore, it seems that pH regulation independently of the implemented strategy (control pH or media buffering) could improve the xylose consumption in presence of glucose.

As a considerable fermentation strategy, co-culture of two or more microorganisms has been applied in several bioprocesses to confront the limitations of pure strains, like biofuels or food industries (Bader et al., 2010). Some examples are the consolidated bioprocessing (CBP) to combine cellulolytic bacteria and another microorganisms, such as, lactic acid bacteria (Shahab et al., 2018) or the co-culture with amylolytic and ethanol fermentation microorganisms to develop competitive simultaneous saccharification and fermentation (SSF) (Lee et al., 2012; Verma et al., 2000). Recently, co-culture of *S. cerevisiae* and *Clostridium* species have gained interest. The metabolic abilities of the yeast could contribute to increase monosaccharide uptake and biofuel production (butanol and ethanol) at the co-culture with *Clostridium* species. The better outcome of the fermentation may be due to the yeast ability to secrete amino-acids to the fermentation media (Luo et al., 2015; Wu et al., 2019). Some studies have shown that co-culture of *S. cerevisiae* and *C. acetobutylicum* improve butanol production from starch-based media (Luo et al., 2017a, 2015), although its

application to lignocellulosic waste remains unexplored. For instance, Qi et al. (2018) were able to increase ABE production from 17.66 to 42.56 g L^{-1} by using a co-culture with *S. cerevisiae* and *C. acetobutylicum* CH02 over the *Clostridium* monoculture when 150 g L^{-1} of cassava was fermented.

The main aim of this work was to evaluate the combination of pH regulation and a co-culture system of *C. acetobutylicum* and *S. cerevisiae* with the target to improve solvent concentrations and sugar uptake from RS hydrolysate. Initially, it was evaluated the effect of the pH control in the exploitation of model substrates (glucose and xylose) by *C. acetobutylicum* for butanol production by using two buffering components (acetate and carbonate). The effect of the best buffering component was further tested with hydrolysates from alkaline-pretreated RS in order to define the pH regulation strategy. Once pH regulation was stablished, the co-culture system of *S. cerevisiae* and *C. acetobutylicum* was studied by using model substrates. The overall strategy was further validated by using alkaline pretreated RS hydrolysates. This work is expected to contribute to enhance solvent concentrations and sugars consumption in fermentations from lignocellulosic biomass.

8.2. Material and methods

8.2.1. Microorganisms, fermentation media and chemical reagents

C. acetobutylicum DSM 792 was purchased from DSMZ, Germany (German Collection of Microorganisms and Cell Cultures). The culture was stored in 20% glycerol at -80 ºC. Prior to the experiments, the microorganism was cultured statically at 37º in 19 g L^{-1} of Reinforced Clostridial Medium (RCM) fortified with 10 g L^{-1} of glucose as seed inoculum. *S. cerevisiae* EYS4 was maintained on YPD agar (yeast extract, 10 g L^{-1}, peptone 20 g L^{-1}, glucose 20 g L^{-1} and agar, 20 g L^{-1}) at 4 ºC. The yeast seed culture was inoculated into 50 ml of YPD broth and incubated at 37 ºC and 150 rpm. The fermentation medium composition was (g L^{-1}): sugars (glucose, xylose or hydrolysate); Yeast Extract, 5; K_2HPO_4, 0.5; KH_2PO_4, 0.5; NH_4Cl, 2; $MgSO_4.7H_2O$, 0.2; $MnSO_4.7H_2O$, 0.01; $FeSO_4.7H_2O$, 0.05; resazurin sodium salt, 0.001 and Antifoam 204, 0.01%. Two different media formulations were tested. Acetate buffer (CH_3COONH_4, 2.2 g L^{-1}) replaced the NH_4Cl of the fermentation medium. Carbonate buffer was formulated by adding $CaCO_3$ (2.5 g L^{-1}) to the above mentioned fermentation media. The media was sterilized by autoclave at 121 ºC during 20 min and the metal solution was filter-sterilized by 0.22 μm. Chemicals were obtained from VWR, except for antifoam 204 (Sigma-Aldrich), $CaCO_3$ (Merk) and yeast extract (Alfa Aesar).

8.2.2. RS pretreatment and hydrolysis

The biomass was obtained from local farmers of L'Albufera situated close to Valencia (Spain). The untreated RS composition on dry weight basis was 35.8 ± 2.1% of cellulose, 17.5 ± 1.4% of hemicellulose, 0.1 ± 0.0% of acid soluble lignin, 14.3 ± 0.4% of acid insoluble lignin, 16.7 ± 0.1% of ash and 15.6% of others. Prior optimized from a previous study (Valles et al., 2021b), the following conditions were applied to obtain the hydrolysate: dried RS was milled to 0.1 to 2 mm particle size; it was pretreated with 0.75% NaOH and a solid loading of 5% (w/w) at 134 ºC for 40 minutes in an autoclave (MED20, J.P. Selecta, Spain); then dried in an oven at 45 ºC for 24 h prior storage at 4 ºC. Enzymatic hydrolysis was performed with 8% (w/w) solids loading at pH 5.2 with a concentration of 20 FPU g-dw^{-1} (Cellic® CTec2, Novozyme, Denmark) at 50 ºC and 150 rpm for 72 h in an orbital incubator (G25, New brunswick scientific, USA). The hydrolysate was stored at -4 Cº prior use.

8.2.3. Experimental set-up

8.2.3.1. Mono and co-cultures reactors

The reactor fermentations were performed with a working volume of 0.8 L (total volume of 1.1 L). The media was flushed with nitrogen gas for 30-35 minutes before inoculation. A 5% v/v inoculum of *C. acetobutylicum* was used in each experiment. The fermentations were performed at 37 ºC and 120 rpm. For the co-culture experiments, a 5% v/v inoculum of *S. cerevisiae* was used. Two kinds of experiments without and with pH control were performed. A minimum set-point control was carried out with NaOH (3M) to keep the pH above the threshold. The experiments were followed using a Tris-compatible flat pH sensor with LoggerPro software (Vernier, USA). The pH probes were sterilized following the procedure by Qureshi et al. (2018). They were sterilised by submerging them in a 50% ethanol solution (v/v) for 12-24 h. After which, the probes were washed with sterile water. Samples were taken at appropriate time-points to analyse cell growth, sugars, and products (butyric acid, acetic acid, butanol, acetone, and ethanol).

8.2.3.2. Co-culture pre-screening

Fermentations were performed in 50-mL serum bottles with 40 mL of working volume; the inoculation was carried out with 5% v/v of *C. acetobutylicum* and then 5% v/v of *S. cerevisiae* at three time lags (0, 5 and 10 h). Monocultures of *C. acetobutylicum* and *S. cerevisiae* were performed as controls. Anaerobic conditions were obtained by

sparging nitrogen in the fermentation medium. The fermentations were carried out in an orbital incubator at 37 ºC and 150 rpm by duplicate. Butanol production was selected as the criteria for stablishing the inoculation procedure of the co-culture reactors.

8.2.4. Experimental plan

Five sets (runs) of experiments were carried out to develop a combined regulation pH strategy with a co-culture fermentation of *C. acetobutylicum* and *S. cerevisiae*. All runs were performed without and with pH control. The value of the minimum pH (4.8) was selected from a prior study by using the same glucose concentration and 5 g L^{-1} carbonate (Capilla et al., 2021). Run 1 was performed with 60 g L^{-1} of glucose to determine the best buffer composition for the two formulations. From these results, ammonium acetate was selected as the best buffer alternative. For the rest of the experiments, two pH regulations were employed: (1) ammonium acetate dosage; or (2) ammonium acetate dosage combined with minimum NaOH pH control. The effect of the pH regulation on xylose in presence of glucose was assessed (run 2). Run 3 was performed by replacing the synthetic substrate with alkali pretreated RS as described in Section 2.2. In run 4, a model concept of co-culture was developed using a synthetic mixture of 35:15 glucose:xylose mimicking RS hydrolysate. In this case, *S. cerevisiae* was inoculated at a selected time after *C. acetobutylicum* according to results derived from Section 3.2.1. Once the co-culture model was established, the effect on solvent production was checked by using RS hydrolysate (run 5). Runs 3 to 5 were performed by duplicate.

8.2.5. Analytical methods

The fermentations were monitored by sampling at desired time points. Cell biomass was determined using an UV-Vis spectrophotometer (SpectroFlex 6600, WTW) at 600 nm (OD_{600}). Biomass concentration of *C. acetobutylicum* (gDM L^{-1}) was estimated by using gDM L^{-1} = 0.2941·OD_{600} + 0.0331 (R^2 = 0.9908*).* Samples were centrifuged at 10000 rpm for 5 minutes (MEGA Star 3.0, VWR, Germany) and filtered by 0.22 µm for analysis. Sugars and products were determined by liquid chromatography (Agilent 1100 Series HPLC system, Agilent Technologies, USA) using a refractive index detector (RID) and diode array detector (DAD) with an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA). The system was operated at 50 ºC. A refractive index detector (RID) was used to detect sugars, butanol, and ethanol, while a diode array detector (DAD) was employed at 210 nm to detect acetic, butyric and levulinic acids and at 280 nm to detect acetone, furfural and 5-(hydroxymethyl)furfural (5-HMF). The mobile

phase was 5 mM of sulphuric acid with a flow of 0.6 ml min^{-1}. The RID was kept at 35 ºC. The running time of the analysis was 45 minutes. The Folin-Denis method was used to quantify the total phenolic compounds expressed as gallic acid equivalents (Folin and Denis, 1912).The values of pKa of acetic acid (4.76) and butyric acid (4.82) were used with the Henderson-Hasselbalch equation (Equation 8.1) to obtain the concentration of undissociated acids.

$$pH = pK_a + log\frac{[A^-]}{[HA]} \quad (8.1)$$

The glucose consumption rate was estimated at the exponential growth phase by Equation 8.2:

$$-q_{glucose} = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \quad (8.2)$$

Where $-q_{glucose}$ corresponds to the glucose consumption rate (g L^{-1} h^{-1}), S_{t1} and S_{t2} are the monosaccharide concentrations at the starting and ending point of the exponential growth phase (g L^{-1}), t_2 and t_1 are the times at the beginning and end of the exponential growth phase (h).

8.3. Results and discussion

8.3.1. Solvent production from RS hydrolysate by *C. acetobutylicum*

8.3.1.1. Effect of the media buffer using glucose as model substrate

The influence of the buffer species on butanol production from glucose (60 g L^{-1}) as main substrate in RS hydrolysate was evaluated by using two alternative components, CH_3COONH_4 or $CaCO_3$, added to the buffering phosphate species (Run 1). Main representative parameters of the ABE fermentation experiments without and with pH control (pH_{min}>4.8) are summarized in Table 8.1. As it can be seen, the reactors without pH control showed a different behaviour depending on the buffer compound. A butanol production of 11.2 g L^{-1} was achieved when acetate was used but only 3.2 g L^{-1} were obtained with carbonate. ABE production also differed, being 19.2 g L^{-1} with acetate and 5.9 g L^{-1} with carbonate. The substantial difference in solvent production with acetate compared with carbonate was related to the glucose consumption in both reactors. In that sense, the glucose was completely depleted (in less than 50 h) with acetate while the carbonate reactor only consumed 55% of the reducing sugar. Therefore, higher butanol yield was also observed (0.199 g g^{-1} for acetate and 0.093 g g^{-1} for carbonate).

The pH recovery was higher for acetate (4.18 to 4.84) than for carbonate (4.57 to 4.71), which was connected to a better development of ABE fermentation when using acetate instead of carbonate. The nearly double glucose consumption rate with carbonate ($-q_{glucose}$: 1.80 g L^{-1} h^{-1}) seemed to impact adversely on the solventogenesis. Indeed, the level of $CaCO_3$ (2.5 g L^{-1}) was not sufficient to prevent the acid crash phenomenon. The total undissociated acid species were higher than 60 mM, the referenced threshold for *Clostridium* species (Maddox et al., 2000). The lower performance of the reactor with carbonate over the reactor with acetate could be explained by the differences in the maximum butyric acid concentration. The production of acids can be modulated by changing the fermentation media; for example, calcium carbonate can enhance the production of acids in ABE fermentation. Ren et al. (2010) observed an increment of ~1.3-fold when fermenting with P2 media over P2 media supplemented with calcium carbonate with *C. acetobutylicum* ATCC 824. Similarly, the reactor with acetate reached 2.87 g L^{-1} of butyric acid (Figure 8.1a), while the reactor with carbonate reached 4.88 g L^{-1} (Data not shown), thus showing an ~1.7-fold increase when the calcium carbonate is used to buffer the fermentation. These results indicated the effect of buffer media formulation on *C. acetobutylicum* DSM 792 metabolism. In this way, Luo et al. (2019) obtained 1.2 g L^{-1} of butanol when fermenting with 0.75 g of K_2HPO_4 and KH_2PO_4 as media buffer, showing that the usage of sole phosphate species as buffer failed to produce enough butanol. Higher level of carbonate (5 g L^{-1}) allowed to achieve better glucose consumption without pH control (Capilla et al., 2021; Raganati et al., 2015), although acid crash was reported in some extent (Capilla et al., 2021).

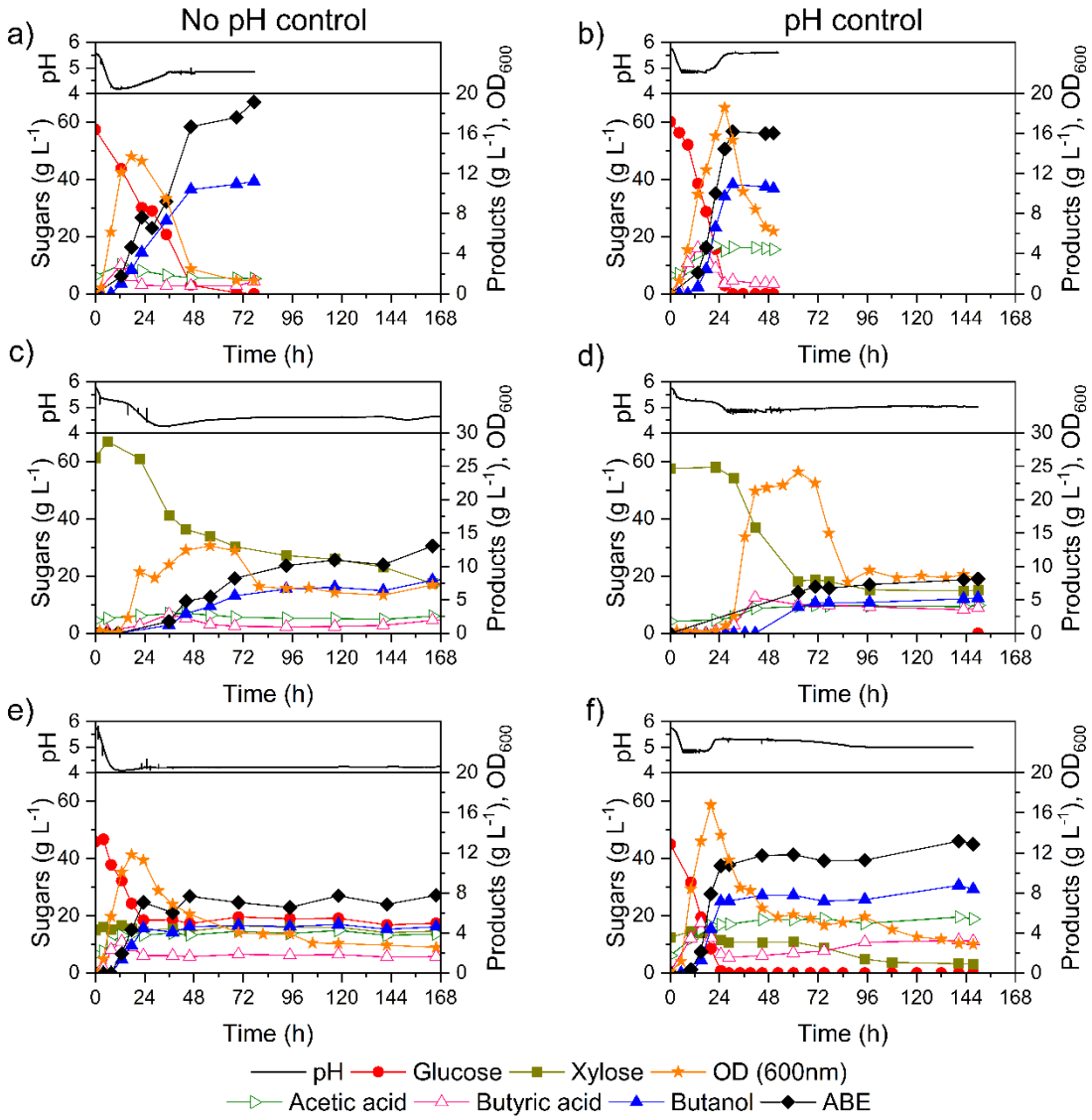

Figure 8.1. ABE fermentation profiles by *C. acetobutylicum* DSM 792. Glucose (60 g L^{-1}): a, no pH control; b, pH control. Xylose (60 g L^{-1}): c, no pH control; d, pH control. Glucose:xylose (45:15 g L^{-1}): e, no pH control; f, pH control

Table 8.1. Solvent production, stoichiometric and kinetic parameters under different buffer formulations with glucose (60 g L^{-1}) as carbon source. Yields were estimated with the maximum solvent concentration. Glucose conversion was calculated at the end of the fermentation.

	Buffer	Butanol $_{max}$ (g L^{-1})	ABE $_{max}$ (g L^{-1})	$Y_{B/S}$ (g g^{-1})	$Y_{ABE/S}$ (g g^{-1})	Gluc. conv. (%)	$-q_{glucose}$ (g L^{-1} h^{-1})	pH $_{min}$	pH $_{final}$
No pH control	Ammonium acetate (2.2 g L^{-1})	11.2	19.2	0.199	0.334	100	0.96	4.18	4.84
	Calcium carbonate (2.5 g L^{-1})	3.2	5.8	0.093	0.172	55	1.80	4.57	4.71
pH control (pH_{min}>4.8)	Ammonium acetate (2.2 g L^{-1})	10.9	16.2	0.181	0.269	100	2.67	4.80	5.58
	Calcium carbonate (2.5 g L^{-1})	9.8	15.1	0.172	0.264	100	1.90	4.80	5.33

Gluco. conv. denotes glucose conversion

The pH regulation had an impact on the switch between acidogenesis and solventogenesis of *C. acetobutylicum* DSM 792 independently of the media buffer. For the two assays, the pH regulation was activated at early times (6 – 7 h) when acids production occurred, thus allowing a good pH recovery in both reactors after exponential growth phase ended, with final pHs>5.3. Respecting the reactor with the acetate buffer, the final butanol concentration was very similar to the uncontrolled counterpart (10.9 over 11.2 g L^{-1}) despite the reduction in ABE solvents (16.2 over 19.2 g L^{-1}). In this case, ABE composition shifted to lower acetone proportion (6.1:3.2:0.7 butanol:acetone:ethanol), closer to the theoretical ratio (6:3:1), compared with the non-pH control reactors (acetate: 5.1:3.8:1.0; carbonate: 4.6:3.7:1.7). Furthermore, butanol yields were similar (>0.18 g g^{-1}), showing that no butanol inhibition occurred when using acetate buffer without or with minimum pH control. Interestingly, the glucose consumption rate with ammonium acetate increased in ~2.8-fold with pH regulation (2.67 g L^{-1} h^{-1}), being the highest achieved over all the experiments. The fast sugar consumption rate shortened the fermentation time from ~50 to ~30 h. Therefore, a substantially increase in butanol productivity was achieved. The pH control enhanced glucose consumption rate with other *Clostridium* species such as *C. beijerinckii* IB4. A higher glucose consumption rate (1.67 ± 0.05 g L^{-1} h^{-1}) was achieved when pH was controlled (pH at 5.5 after reached) versus no pH control (1.03 ± 0.04 g L^{-1} h^{-1}) (M. Jiang et al., 2014). In the case of using carbonate, the

pH regulation impacted favourably on the solvent production and glucose depletion, but not in the glucose consumption rate. The complete depletion of glucose led to an increment in butanol (ABE) production from 3.2 (5.9) to 9.8 (15.1) g L^{-1} (Table 8.1), while butanol yield (0.172 g g^{-1}) was similar to that of the fermenter with acetate buffer. Furthermore, pH regulation at early stages was shown as a very efficient strategy to avoid acid crash comparing with making changes on media formulation. Moreover, M. Jiang et al., (2014) observed that delaying the pH control more over 24 hours of the fermentation did not avoid acid crash. Our approach, based on fixing a minimum pH rather than stablishing a specific time to start pH control, allows well-fitting the pH regulation during the exponential growth phase independently of the growth kinetics. In our case, both buffers exhibited good performance in solvent production when active pH control was used. The main difference between them was in the glucose rate, being 40% higher in the case of using acetate. Consequently, the higher glucose consumption rate with ammonium acetate led to a greater productivity over the use of calcium carbonate while reducing the fermentation time. Moreover, without pH regulation the use of 2.2 g L^{-1} of ammonium acetate does not exhibit acid crash leading to better solvent production than 2.5 g L^{-1} of calcium carbonate. Thus, by the criteria of incrementing the solvent productivity (with pH regulation) or overcome acid crash (without pH regulation), subsequent experiments were carried out with ammonium acetate as media buffer.

8.3.1.2. Effect of the pH regulation on xylose and mixtures glucose:xylose

In order to evaluate the exploitation of lignocellulosic waste onto ABE solvents, the effect of the pH regulation on xylose (secondary reducing sugar) consumption under the presence of glucose (primary reducing sugar) was studied. Two sets of experiments were performed by keeping constant the reducing sugar concentration at 60 g L^{-1} and in absence or presence of glucose (Run 2). The selected mixture was of 45:15 g L^{-1} glucose: xylose which is on the typical proportion between the two monosaccharides in hydrolysates from lignocellulosic waste. Fermentation profiles with uncontrolled pH and controlled pH using xylose and with a mixture of glucose:xylose were compiled in Figure 8.1c-f. For comparison purposes, patterns with pure glucose and same media formulation (2.2 g L^{-1} acetate, Table 8.1) were also depicted (Figure 8.1a-b). From the reactors with pure xylose (Figure 8.1c-d), it can be seen a lag-phase of approximately 24 h not observed previously with glucose (Figure 8.1a-b). This is linked to the change of the carbon source from the inoculum growth (RCM with glucose). Without pH control, similar sugar consumption rates were achieved during exponential growth phase,

independently of the monosaccharide (glucose: 0.96; xylose: 1.13 g L^{-1} h^{-1}). Nevertheless, xylose consumption declined from 48 h when butanol concentration started to increase (Figure 8.1c) while no changes on consumption were observed with glucose. This reduction in xylose uptake would be related to product inhibition. In this sense, a butanol concentration of ~8.0 g L^{-1} had been reported as inhibitory when using xylose as carbon source (Ounine et al., 1985). At the end of the fermentation, only 72% of xylose was consumed, whereas butanol production reached the inhibitory value (8.0 g L^{-1}; butanol yield of 0.181 g g^{-1}). On the other hand, the fermentation of the sugar mixture (45:15) without pH control stopped at 24h due to an acid crash phenomenon (Figure 8.1e). Nevertheless, a butanol (ABE) production of 4.9 (7.7) g L^{-1} was achieved from a glucose consumption of 63%, with a butanol (ABE) yield of 0.182 (0.289) g g^{-1}. Glucose consumption rate, with a 25% lower initial glucose level, was faster in the mixture (1.35 g L^{-1} h^{-1}) than with pure glucose (0.96 g L^{-1} h^{-1}). However, was not sufficiently high to promote the solventogenesis shift before acid crash occurred. Results indicate that a minimum level of initial glucose would be required for completion of ABE fermentation with this strain when pH evolved spontaneously.

A similar xylose consumption rate was observed with and without pH control; however, the pH control caused a carbon flux redistribution to boost biomass production (Figure 8.1d). A OD_{600} ~1.6-fold higher was achieved (at the maximum point) when using pure xylose comparing with the use of glucose as carbon source (xylose: 24.2 or 7.2 g L^{-1} at ~62 h; glucose:14.9 or 4.4 g L^{-1} at ~26 h). We speculate that the metabolism shift towards cell synthesis was associated to the availability of organic nitrogen from yeast extract (5 g L^{-1}). W. Jiang et al. (2014) observed some increase in biomass growth with *C. acetobutylicum* ATCC 824 under pH control after the acidogenic phase with same yeast extract concentration, which was accompanied by an improvement of solvent production. However, contrarily to them we observed a decrease in butanol (ABE) production from 8.0 (13.1) g L^{-1} without pH control to 5.3 (8.2) g L^{-1} with pH control at 4.8 (Figure 8.1c-d), probably due to the excessive biomass growth achieved in our study. In any case, data shown that xylose will be consumed after glucose exhaustion when using lignocellulosic hydrolysates. In the sugar mixture experiment, 60 h were required to start xylose consumption after glucose depletion. In this regard, the utilization of pH control with the glucose:xylose mixture allowed avoiding acid crash, thus enhancing the full glucose conversion in <24 h ($-q_{glucose}$: 2.38 g L^{-1} h^{-1}), and favoring the further xylose uptake without an excessive biomass growth (Figure 8.1f). Although the pH regulation was unable to avoid the CCR phenomena, positively this delay on xylose consumption did not adversely impact on the butanol (ABE) production, which was 8.8 (13.2) g L^{-1}.

Indeed, solvent production was quite close to the estimated values from the experimental yields with solely monosaccharides under pH control (butanol: 9.3; ABE: 13.9 g L^{-1}). As conclusion, ABE fermentation with *C. acetobutylicum* DSM 792 benefits for pH control to efficiently produced ABE solvents from glucose and xylose mixtures. Moreover, the implementation of in-situ product recovery techniques would improve even more the xylose uptake in presence of glucose by avoiding butanol inhibitory levels.

8.3.1.3. The pH validation strategy using RS hydrolysates

The pH regulation strategy was validated by replacing the model substrates by the alkali-pretreated RS hydrolysate (Run 3, Figure 8.2). The hydrolysate characteristics at the final medium can be observed in Table 8.2. At the beginning of the fermentation reducing sugar concentration was 34.0 ± 0.7 g L^{-1} of glucose and 14.0 ± 0.6 g L^{-1} of xylose. In addition, the initial acetic acid was 4.9 ± 0.7 g L^{-1}. The cellobiose and arabinose remained nearly unchanged through the experiment. The total concentration of phenolic compounds was 0.13 ± 0.01 g L^{-1}. It was expected from the assay with the mixture model (Figure 8.1e) the lack of pH control caused an acid crash stopping the fermentation at 24 h (Figure 8.2a), hence corroborating that low initial levels of glucose require of pH control for ABE fermentation with this strain. Under pH control, a butanol (ABE) production of 6.5 ± 0.1 (9.5 ± 0.8) g L^{-1} was achieved at the end of the fermentation. The pH control favoured the consumption of xylose after glucose was depleted (47 ± 10%). Some xylose remaining as final butanol approached to inhibitory levels. In addition, butanol and ABE yields (butanol: 0.167 ± 0.005, ABE: 0.261 ± 0.002 g g^{-1}) were slightly better over the synthetic mixture (butanol: 0.161, ABE: 0.243 g g^{-1}). The complex composition of the RS hydrolysate better employed the sugar content and mitigated the CCR phenomena when the pH control was applied. In fact, the butanol (ABE) predicted concentrations from the results obtained with the synthetic media were 7.0 (10.5) g L^{-1}, similar to the experimental productions obtained in the assay (6.5 (9.5) g L^{-1}), thus validating the pH control strategy in terms of solvent production. The glucose consumption rates were 0.96 ± 0.13 and 2.38 ± 0.02 g L^{-1} h^{-1} for no pH and pH-controlled fermentations, respectively. Similar values were obtained with synthetic (pure glucose and sugar mixture). Hence, demonstrating no inhibition of glucose uptake occurred when the RS hydrolysate is fermented. The production herein obtained are among the referenced concentrations. For example, Amiri et al. (2014) produced 7.1 (10.5) g L^{-1} of butanol (ABE) by using organosolv pretreated RS with *C. acetobutylicum* NRRL B-591. Other *Clostridium* species can be also used; in this sense, Valles et al. (2021) achieved

a higher butanol (ABE) concentration of 10.1 (16.7) g L^{-1} with alkali pretreated RS using *C. beijerinckii* DSM 6422.

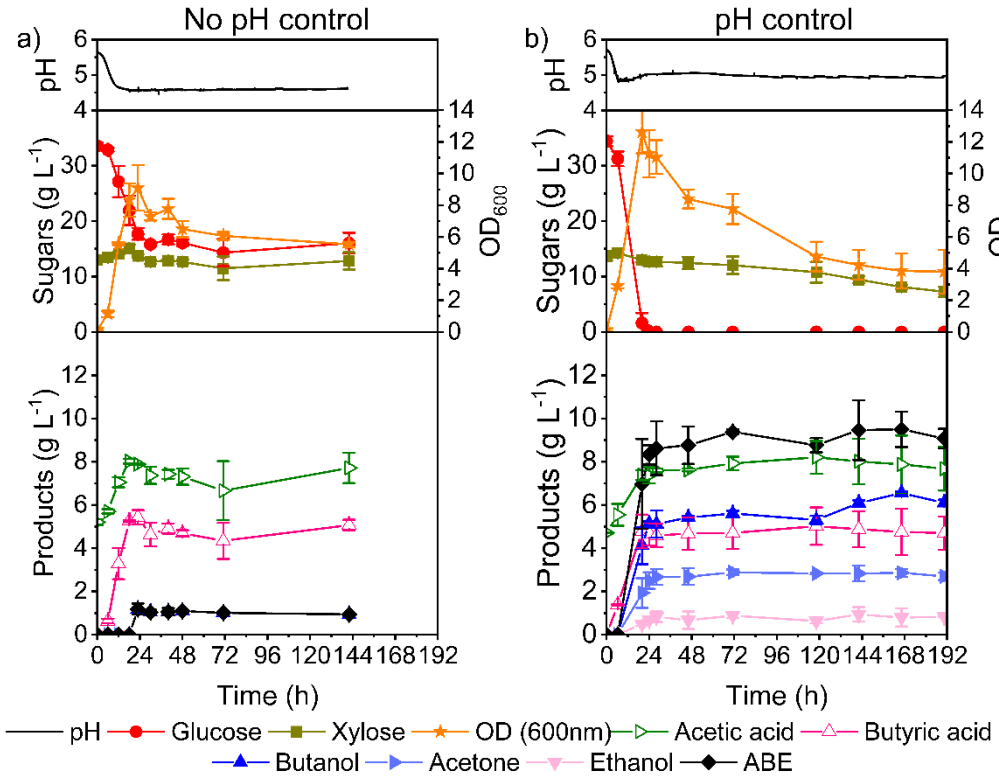

Figure 8.2. ABE fermentation profiles by *C. acetobutylicum* DSM 792 with RS hydrolysate. a, no pH control and b, pH control

Table 8.2. Hydrolysate characteristics in the fermentation medium.

Compound	**Concentration (g L^{-1})**
Glucose	34.0 ± 0.7
Xylose	13.0 ± 0.6
Cellobiose	4.5 ± 0.2
Arabinose	1.5 ± 0.1
Acetic acid	4.9 ± 0.7
Phenolic compounds	0.13 ± 0.01
Levulinic acid	N/D
Furfural	N/D
5-HMF	N/D

N/D denotes no detected.

8.3.2. Solvents production by co-culture of *C. acetobutylicum* and *S. cerevisiae*

8.3.2.1. Effect of time inoculation

Prior to evaluate the effect of pH regulation on the co-culture of *C. acetobutylicum* DSM 792 and *S. cerevisiae* EYS4, it was stablished the elapse time between inoculations of both species to promote butanol production. A screening experiment with 50-mL serum bottles was carried out by inoculating *S. cerevisiae* at 0, 5 and 10 h after *C. acetobutylicum* inoculation. Initial glucose and xylose levels mimicked the RS hydrolysate concentration (35 and 15 g L^{-1} of glucose and xylose). To analyse the effect of inoculation time on the substrate competition between the two species, and hence on the solvent redistribution, solvent production at early-stage (24 h) was considered. Solvent production and glucose consumption rate at 24 h of fermentation along with the remaining glucose at 10 h were summarized in Table 8.3. The butanol production at 24 h increased in all co-culture experiments compared with the *Clostridium* monoculture (Table 8.3). Moreover, ABE production increased due to ethanol production by *S. cerevisiae*. In fact, the inoculation of the yeast simultaneously to *C. acetobutylicum* (0 h) led to mainly ethanol synthesis (ethanol/butanol ratio: 3.4 ± 0.1). The earlier presence of the yeast (0 h) caused a very early competition from the available sugar in the fermentation media between the two species, adversely impacting on butanol production. By postponing 5 h its inoculation the ethanol/butanol ratio decreased drastically (ethanol/butanol ratio: 1.1 ± 0.1), indicating that the conversion of glucose to acids by *Clostridium* was not hindered by *S. cerevisiae* growth. Further delay on the yeast inoculation (10 h) did not improve butanol proportion (ethanol/butanol ratio: 1.2 ± 0.2) while decreasing the overall glucose consumption rate. Although, yeast inoculation time at 0 h gave better results in terms of ABE production, it was decided to delay 5 h the inoculation time of the yeast from the bacteria inoculation to promote butanol production over ethanol.

8.3.2.1. Effect of pH regulation on the co-culture

Batch reactors without pH control and by controlling pH_{min}>4.8 were performed for co-culture fermentations using *C. acetobutylicum* and *S. cerevisiae*. The inoculation of *S. cerevisiae* was performed at 5 h after the beginning of the fermentation as indicated previously. The composition of the synthetic media (glucose: 35, xylose: 15 g L^{-1}, Run 4) was equal to the concentration of the major sugars on the alkali pretreated RS hydrolysate (Table 8.2), having a slightly lower content in glucose than the synthetic mixture used in previous experiments with the solely *Clostridium* strain (glucose: 45,

xylose: 15 g L^{-1}). The co-culture fermentation patterns are depicted in Figure 8.3. Regarding the uncontrolled pH experiment (Figure 8.3a), it was observed the same acid crash phenomenon as without the use of *S. cerevisiae* (Figure 8.1e); the pH decreased rapidly to 4.0, so glucose was not completely exhausted. However, a higher ABE production was achieved (9.3 ± 0.2 g L^{-1} g L^{-1} versus 7.7 g L^{-1}), indicating the beneficial effect by the presence of the yeast to improve the overall glucose uptake (from 66% to 83 ± 5%), even if an early acid crash occurred. Glucose consumption rate was also higher (1.65 ± 0.04 g L^{-1} h^{-1}) compared to the monoculture (1.35 g L^{-1} h^{-1}). The increment in solvents was mainly due to the increase in ethanol production by the alcoholic fermentation of *S. cerevisiae* (from 0.3 to 3.4 g L^{-1}). Butanol production versus total available glucose was only slightly higher in presence of the yeast than in its absence, as 4.0 ± 0.6 g L^{-1} of butanol with 35 g L^{-1} of glucose for the co-culture was obtained compared with 4.9 g L^{-1} of butanol with 45 g L^{-1} for the monoculture. In any case, these results showed that independently of the presence of the yeast, pH regulation is required for a properly development of the *C. acetobutylicum* metabolism.

The profiles of sugar consumption and acid and solvents production during the co-culture fermentation under pH control are shown in Figure 8.3b. The total solvent production was 10.9 ± 0.6 g L^{-1}, from which butanol was 6.2 ± 0.8 g L^{-1}. Unsuccessfully, ABE production versus total available sugar was equal to that observed with the solely *Clostridium* assay. Nevertheless, ABE composition slightly shifted to more ethanol ratio due to the competition of both species for the glucose. The reduction on butanol production considering the potential production if all sugars were metabolized by the *Clostridium* strain was of 1.2 g L^{-1}. Qi et al. (2015) tested several combinations of elapsing times between inoculation of *C. acetobutylicum* and *S. cerevisiae* by using a mixture of 25:25 glucose:xylose. As we also observed, test butanol concentration was not improved in any of the co-culture assays comparing with the mono-*Clostridium* test. The best results in terms of ABE production obtained by these authors corresponded to the initial inoculation of *S. cerevisiae* followed by inoculation 24 h after *C. acetobutylicum.* By using this strategy, these authors slightly improved ABE production from 18.45 g L^{-1} (solely *C. acetobutylicum*) to 19.62 g L^{-1}. However, a drastic reduction in butanol production (5.29 *vs* 11.22 g L^{-1}) was given due to the faster metabolic rate of the yeast. In terms of butanol production, the delay in the yeast inoculation seems a better strategy.

Table 8.3. Effect of inoculation time of *S. cerevisiae* on solvent production (at 24 h) for the co-culture system. Remaining glucose at 10 and 24 h are depicted. The ethanol/butanol ratio (E/B ratio) and the glucose consumption rate were calculated at 24 h.

Experiment	Yeast inoculation time (h)	Butanol (g L^{-1})	Ethanol (g L^{-1})	ABE (g L^{-1})	Ethanol/Butanol ratio	$Glucose_{10\ h}$ (g L^{-1})	$-q_{glucose}$ (g L^{-1} h^{-1})
C. acetobutylicum mono-culture	N/A	1.6 ± 0.3	0.2 ± 0.1	2.9 ± 0.5	0.1 ± 0.0	14.9 ± 0.2	0.72 ± 0.01
S. cerevisiae mono-culture	N/A	0.0 ± 0.0	16.5 ± 0.4	16.5 ± 0.4	N/A	7.5 ± 2.4	1.17 ± 0.02
Co-culture	0	2.0 ± 0.3	6.8 ± 1.1	10.0 ± 1.6	3.4 ± 0.1	6.6 ± 3.2	1.12 ± 0.00
Co-culture	5	2.0 ± 0.2	2.3 ± 0.0	5.3 ± 0.3	1.1 ± 0.1	12.2 ± 1.1	0.96 ± 0.02
Co-culture	10	1.8 ± 0.1	2.1 ± 0.3	4.9 ± 0.2	1.2 ± 0.2	13.4 ± 0.2	0.91 ± 0.02

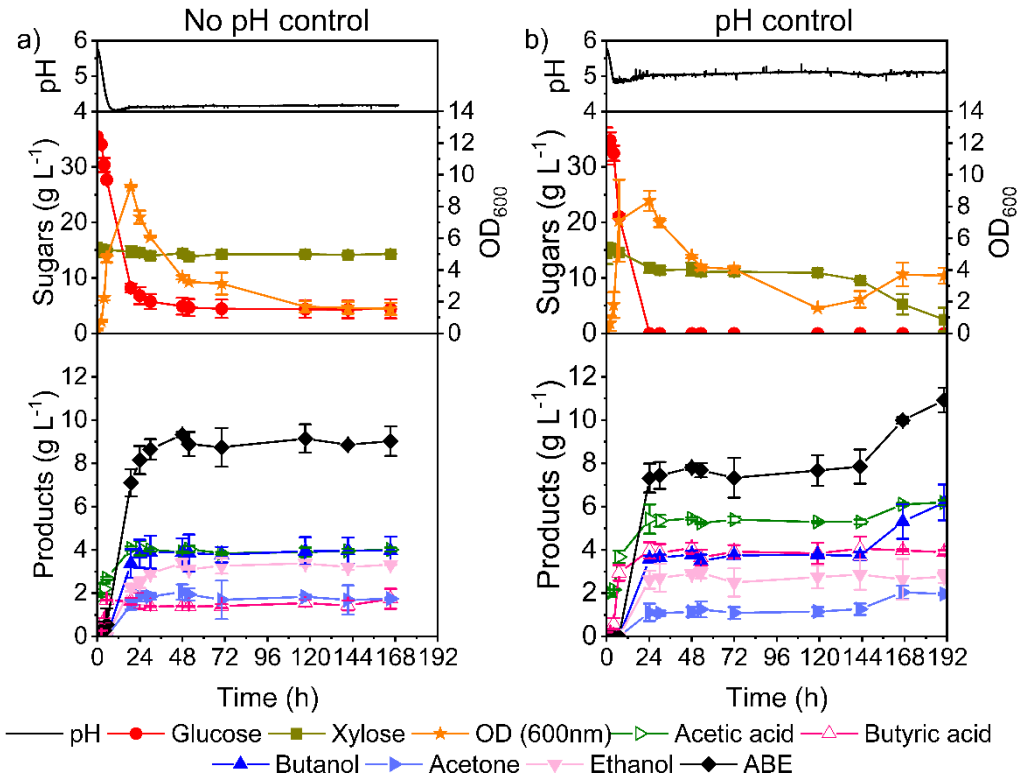

Figure 8.3. Co-culture fermentation profiles by *C. acetobutylicum* DSM 792 and *S. cerevisiae* EYS4 with synthetic media (glucose: 35; xylose: 15 g L^{-1}). a, no pH control; b, pH control

Although the presence of the yeast did not reduce the adaptation time of the *Clostridium* strain to start xylose consumption, it enhanced the xylose consumption up to 85 ± 13%. Positively, it was accompanied by a simultaneously increase of butanol up to 2.4 ± 0.8 g L^{-1} and with some acetone production (0.8 ± 0.2 g L^{-1}). The beneficial effect on the conversion of xylose to butanol by *C. acetobutylicum* would be linked to the ability of *S. cerevisiae* to secrete amino acids which could be uptake by the *Clostridium* species. In this regard, Wu et al. (2019) monitored the secreted amino-acids (aspartic, aliphatic and aromatic acids family and arginine) by *S. cerevisiae* in a co-culture system with *C. beijerinckii*, observing, for example, an increment of 150% for phenylalanine concentration over the *Clostridium* monoculture. The assimilation of amino-acids by *Clostridium* species would increase monosaccharides intracellular transportation and butanol tolerance (Luo et al., 2015; Wu et al., 2019). This evidence opened to test the co-culture system in more realistic conditions.

The profiles of sugar utilization and product formation during the co-culture fermentation of alkali pretreated RS hydrolysate (Run 5) are shown in Figure 8.4. As in the previous experiments occurred, acid crash impeded an adequate development of the solventogenesis under lack of pH control (Figure 8.4a), corroborating the importance of

controlling pH in such system. By controlling the pH, it was achieved the highest solvent production among the experiments with the same initial sugar levels (ABE: 13.1 ± 0.1, from which butanol: 7.0 ± 0.4, acetone: 3.4 ± 0.2 and ethanol: 2.7 ± 0.7 g L^{-1}). Successfully, the *S. cerevisiae* and *C. acetobutylicum* co-culture was able to increase not only ABE, but also slightly the butanol production. The enhancement of the ethanol production (~3.0-fold) in this co-culture fermentation is of importance for the better exploitation of the RS hydrolysate. The use of alkali RS hydrolysate in the co-culture alleviated in some extent the lag phase of the xylose metabolism, as the consumption started at ~72 h instead of ~120 h. At the end of the fermentation, the maximum xylose uptake (94 ± 1%) was detected, which was ~2.0-fold over the RS hydrolysate monoculture. At the same time, ABE production increased suddenly up to 4.4 ± 0.1 (butanol: 2.7 ± 0.2; acetone: 1.3 ± 0.0; ethanol: 0.4 ± 0.3) g L^{-1}. The better results in comparison with the synthetic media can be associated to the complex chemical composition of the RS hydrolysate. In this sense, Jin et al. (2019) observed a faster sugar consumption when fermenting apple pomace residue compared with using a sugar solution; furthermore, Moradi et al. (2013) produced more butanol when fermenting with alkali pretreated RS hydrolysate over a pure sugars medium. Literature regarding the utilization of co-cultures with *Clostridium* and *S. cerevisiae* is still scarce and most of them used starchy substrates (Table 8.4). In our case, the combination of an adequate pH regulation and the co-culture system with RS hydrolysate substantially boosted the consumption of xylose, which allowed the better use of the substrate (94% over the 47% of the monoculture). This led to an increase on ABE production of about 38% (Table 8.4). The ABE yield remained similar due to simultaneous increment in ABE production and sugar consumption. Maximum ABE productivity remained constant over the *Clostridium* monoculture as it was associated to glucose. The use of co-cultures with starchy substrates up to 150 g L^{-1} also exhibited an increment in total ABE production in a range of 37 to 116% over its monocultures (Table 8.4) (Luo et al., 2017b, 2015; Qi et al., 2018). These easier assimilable residues composed mainly by glucose led to increments in either the ABE yield (Qi et al., 2018) or the productivities (Luo et al., 2017b, 2015) (Table 8.4). In the sense of lignocellulosic substrates, it seems that the *Clostridium* species has a significant impact. Using *C. beijerinckii* the butanol production increased from 4.22 to 10.62 g L^{-1} by implementing the co-culture system with *S. cerevisiae* (Wu et al., 2020), which was not evident with *C. acetobutylicum*. Our results support that the co-culture strategy with *Clostridium* species and *S. cerevisiae* can extend ABE concentrations not only with starchy substrates, but also with lignocellulosic wastes. Furthermore, this co-culture strategy enhances the exploitation of the substrate.

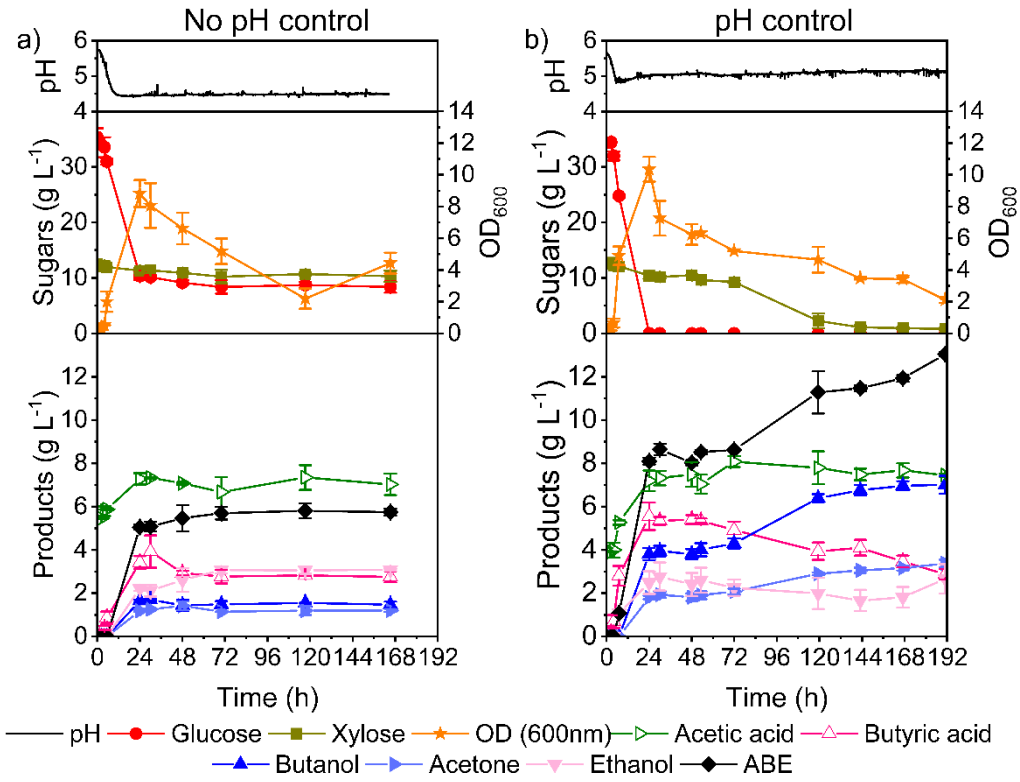

Figure 8.4. Co-culture fermentation profiles by *C. acetobutylicum* DSM 792 and *S. cerevisiae* EYS4 with RS hydrolysate. a, no pH control; b, pH control

Table 8.4. Comparison of production performance from *Clostridium* and *S. cerevisiae* co-cultures.

Substrate/sugar concentration (g L^{-1})	Microorganism	Production (g L^{-1})			Yield (g g^{-1})		Max. Productivity (g L^{-1} h^{-1})		pH control	References
		Butanol	ABE	ABE increment (%)	ABE	ABE increment (%)	ABE	ABE increment (%)		
Rice straw/58	*C. acetobutylicum* DSM 792	6.50	9.50	38	0.24	9	0.35	0	Yes	This study
	C. acetobutylicum DSM 792/ *S. cerevisiae* EYS4	7.00	13.10		0.28		0.35		Yes	
Corn flour /150	*C. acetobutylicum* ATCC 824	11.63	18.77	37	0.37	-2	0.29	66	No	Luo et al. (2015)
	C. acetobutylicum ATCC 824/ *S. cerevisiae*	11.91	25.69		0.36		0.48		Yes	
Corn flour/150	*C. acetobutylicum* ATCC 824	7.67	12.70	47			0.25	31	No	Luo et al. (2017b)
	C. acetobutylicum ATCC 824/ *S. cerevisiae*	9.53	18.61				0.33		No	
Cassava chip flour/150	*C. acetobutylicum* CH02	13.36	19.70	116	0.32	44			No	Qi et al. (2018)
	C. acetobutylicum CH02/ *S. cerevisiae*	11.11	42.56		0.46				No	
Rice straw/~45	*C. beijerinckii* F-6	4.22							No	Wu et al. (2020)
	C. beijerinckii F-6/ *S. cerevisiae*	10.62							No	

8.4. Conclusions

The main aim of this work was to evaluate the integration of pH regulation and a co-culture system of *C. acetobutylicum* and *S. cerevisiae* with the target to improve xylose consumption and subsequent solvent concentrations from RS hydrolysate**.** The strategy of regulating the pH combining ammonium acetate and limiting the at pH_{min}>4.8 was successfully applied to RS hydrolysate. The use of the co-culture of *C. acetobutylicum* and *S. cerevisiae* led to an increase of 1.4-fold of the total solvent concentration, mainly due to ethanol production. Moreover, *S. cerevisiae* promoted the xylose uptake by *C. acetobutylicum*, probably associated due to the amino-acids excreted by the yeast, thus enhancing the overall exploitation of the rice straw. The better exploitation of the secondary monosaccharaide of hydrolysates, xylose, in the co-culture was correlated to butanol production by *Clostridium* species. This co-culture fermentation strategy can be used to increment butanol and ethanol co-production and sugar consumption from lignocellulosic wastes.

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Author contribution

M. Capilla: Investigation, Data curation, Formal analysis, Writing - original draft, Visualization. A. Valles: Conceptualization, Methodology, Visualization. P. San-Valero: Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision. F. J. Álvarez-Hornos: Conceptualization, Methodology, Resources. C. Gabaldón: Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Declarations

Conflict of interests

The authors declare no competing interests.

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9. DEVELOPMENT OF A SINGLE CONTINUOUS STIRRED TANK REACTOR CONFIGURATION WITH *CLOSTRIDIUM CARBOXIDIVORANS* FOR SYNGAS FERMENTATION

As an alternative to the fermentation of rice straw hydrolysate, the direct gasification of the lignocellulosic feedstocks results on syngas mixtures (mainly CO, CO_2 and H_2). These gas streams can be utilized by some Clostridium spp. in order to produce bioalcohols via the HBE fermentation. In this sense, there is a growing interest on exploring C1-gas fermentations with C. carboxidivorans in order to produce bioalcohols. This chapter introduces the development of a continuous free cell fermentation with the microbial strain C. carboxidivorans DSM 15243. The experimental study of HBE fermentation was carried out with model CO substrate to evaluate the effect of pH and dilution rate as process parameters.

A manuscript including some results discussed herein is under preparation in collaboration with the host research group.

DEVELOPMENT OF A SINGLE CONTINUOUS STIRRED TANK REACTOR CONFIGURATION WITH *CLOSTRIDIUM CARBOXIDIVORANS* FOR SYNGAS FERMENTATION

Abstract

The ability of *Clostridium carboxidivorans* for fixing syngas (CO, CO_2 and H_2) has attracted attention to produce bioalcohols via the hexanol-butanol-ethanol (HBE) fermentation. This work aimed to study the HBE fermentation in a continuous stirred tank reactor (CSTR) using *C. carboxidivorans* from pure CO. Two reactors operated at different pH set-points (5.4 or 5.6) were compared at a range of a dilution factors ranging from 0.034 to 0.167 h^{-1} using pure CO. The reactor operated at the pH of 5.6 exhibited >1.8-fold hexanol productivity over the experiment at pH of 5.4 for dilution rates up to 0.050 h^{-1}. The increment to a dilution rate of 0.083 h^{-1} (reactor at 5.6) raised the ethanol productivity by 1.4-fold while maintaining the butanol and hexanol productivity steady over the previous dilution rate of 0.05 h^{-1}. The best hexanol productivity obtained in this work was 138 mg L^{-1} day^{-1} with a pH of 5.6 and a dilution rate of 0.034 h^{-1}, being the highest among literature with CSTR fed with gas currents. Nevertheless, the highest dilution rate tested (0.167 h^{-1}) provoked the cease of the ability to produce hexanol at pH of 5.6. Results corroborate the viability of using single-CSTR configurations for hexanol production using CO as substrate. Further research is needed to validate these results with syngas mixtures.

Keywords: biohexanol, HBE fermentation, CSTR, syngas, pH control

9.1. Introduction

The shifting to a global greener economy arises from the necessity to mitigate the climate change. In this sense, biorefineries using lignocellulosic feedstocks can be developed as an alternative to traditional industrial production of fuels and bioalcohols (IEA-Bioenergy, 2020). The gasification of lignocellulosic waste into syngas (mainly CO, CO_2 and H_2) has several advantages over the strategy based on sugar release. Among the advantages, it is remarkable that the gasification allows the full conversion of the waste into a fermentable carbon source; it saves from high-cost pretreatment steps; and the fermentation itself is less affected by the fluctuations in the gas ratio composition (H_2:CO:CO_2) (Munasinghe and Khanal, 2010). Moreover, some industries, like steel milling or petroleum refining, generate suitable gas streams that could be coupled in a

similar way to the syngas fermentation to valorise the waste gas currents (Abubackar et al., 2011).

Acetogenic bacteria, such as, *C. carboxidivorans* can grow autotrophically using gas streams rich on CO. This microorganism performs the Wood-Ljungdahl pathway fixating C1 gases into acetyl-CoA. This metabolite will be later on employed to generate short and middle carbon chain metabolites (acids-solvents), such as, acetic acid-ethanol (C2, two carbons), butyric acid-butanol (C4) and hexanoic acid-hexanol (C6) via the HBE fermentation (Fernández-Naveira et al., 2017a; Liou et al., 2005). The production of metabolites is sequential on the number of carbons. Thus, the initial metabolites synthetized are acetic acid and ethanol, after which, the carbon chain can be extended to the C4 and C6 metabolites (Kottenhahn et al., 2021). The syngas fermentation still faces some challenges. Among them, the pH is a key parameter for the development of solventogenic *Clostridium* spp. In this sense, *C. carboxidivorans* has an optimum range for growth between 5.0 to 7.0 (Fernández-Naveira et al., 2017a). The fine tuning of the pH can improve the outcome of the HBE fermentation. For instance, a pH of 5.75 resulted in a simultaneous production of acids and solvents when performed in bioreactors with continuous gas-fed (Fernández-Naveira et al., 2016b). Another challenge is the low water solubility of the syngas gases, especially the CO and H_2, which limits the mass transport rate to the fermentation media compared to aerobic processes (Fernández-Naveira et al., 2017a). The configuration of the reactor plays an important role to increase the gas transfer to the fermentation broth. Between fixed and stirred tank reactors, stirred tank configurations are preferred to favour gas-liquid mass transfer with low-water solubility gases. By using CSTRs, gas is constantly supplied through diffusers coupled with the mechanical agitation, letting to split large bubbles into smaller one, thus improving the gas-liquid mass transfer coefficients. Additionally, the regulation of the stirring velocity could also favoured the gas mass transfer to the liquid (Fernández-Naveira et al., 2017a; Vees et al., 2020). Another of the advantages of continuous operation with acetogenic bacteria is that single-stage chemostats have been reported to easily achieve steady-state and have never been descripted to degenerate (Vees et al., 2020).

The production of alcohols, among them the hexanol, with *C. carboxidivorans* in stirred tank reactors has been previously reported. In this regard, the use of a stirred batch reactor with continuous syngas flow at a pH of 4.75 produced a maximum hexanol concentration of 0.72 g L^{-1} in 212 h while at a pH of 5.75 obtained a production of 0.85 g L^{-1} in 310 h (Fernández-Naveira et al., 2017c). Moreover, Rückel et al. (2021) obtained

a hexanol production of 0.16 g L^{-1} in ~80 h with a stirred tank reactor. The employment of CSTR configurations have reported an hexanol production of 0.7 g L^{-1} at a dilution rate of 0.05 h^{-1} with a fixed pH of 6.0 using a mixotrophy feed of glucose (10 g L^{-1}) and CO (20%) (Vees et al., 2022). The separation of the acidogenic and solventogenic phases have also been performed in cascade reactors resulting in an overall hexanol productivity of 80 mg L^{-1} day^{-1} (Doll et al., 2018).

Previous research in the Bioprocess Engineering Laboratory from the *Università degli Studi di Napoli Federico II,* where I carried out my international stay, has been focused on evaluating the behaviour of *C. carboxidivorans* in serum bottles using pure CO as model substrate. In this regard, it has been assessed the effect of the partial pressure of CO and the liquid to gas volume ratio on the HBE fermentation. The CO partial pressure impacted on the growth kinetics, where CO inhibition was found in the range of 0.5-2.5 atm. The best operational CO partial pressure in serum bottles was 1.1 atm, which is equivalent to a CO concentration of ~25 mg L^{-1}. The specific growth rates obtained during these experiments were 0.060 to 0.100 h^{-1} depending on the CO partial pressure and the liquid to gas volume ratio (Lanzillo et al., 2020). Additionally, this research group have developed a growth model for *C. carboxidivorans* under pure CO (Ruggiero et al., 2022). As a step forward to increase the overall bioalcohol productivities, the work detailed herein deals with the development of a CSTR feed with pure CO by using *C. carboxidivorans* DSM 15243. Within this aim, a range of dilution rates was tested under controlled pH continuous HBE fermentation.

9.2. Materials and methods

9.2.1. Microorganism, culture media and chemical reagents

C. carboxidivorans DSM 15243 (strain designation P7) was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). For inoculum propagation prior fermentation, the microorganism was cultured in 12 mL Wilkins Chalgren Anaerobic Broth (WCAB) using 15 mL Hungate tubes. The culture was grown in anaerobic conditions at 35 ºC and 130 rpm for 24 h. The composition of the fermentation medium contained: 1 g L^{-1} yeast extract; 25 mL L^{-1} mineral solution; 10 mL L^{-1} trace metal solution; 10 mL L^{-1} vitamin solution and 0.6 g L^{-1} L-cysteine. The mineral stock solution was composed (per litre) of 100 g NH_4Cl, 80 g NaCl, 10 g KH_2PO_4, 10 g KCl, 20 g $MgSO_4$, and 4 g $CaCl_2$. The vitamin stock solution was composed (per litre) of 10 mg pyridoxine, 5 mg each of calcium pantothenate, thiamine, riboflavin, thioctic acid, nicotinic acid, para-amino benzoic acid, and vitamin

B12, and 2 mg each of D-biotin, 2-mercaptoethane-sulfonic acid and folic acid. The trace metal stock solution was composed (per litre) of 2 g nitrilotriacetic acid, 1 g manganese sulphate, 0.80 g ferrous ammonium sulphate, 0.20 g zinc sulphate, 0.20 g cobalt chloride, and 20 mg each of cupric chloride, sodium molybdate, nickel chloride, sodium selenate, and sodium tungstate. Chemicals were purchased from Sigma Aldrich (Milan, Italy).

9.2.2. HBE fermentation on CSTR experiments

Continuous fermentations were carried out in 250 mL MiniBio bioreactors (Applikon Biotechnology, Delft, Netherlands) using 120 mL of working volume coupled with an Applikon my-Control unit (Applikon Biotechnology, Delft, Netherlands). A scheme of the experimental set-up is shown in Figure 9.1. Fermentation media without vitamin solution and L-cysteine was autoclaved at 121 ºC during 20 min. Anaerobic conditions were obtained by bubbling 100% CO at 10 mL min^{-1} prior 24 h of inoculation. The CO flow was maintained for continuous gas-feeding during the whole experimentation. Prior inoculation, vitamin solution and L-cysteine were added from stock solutions. The vitamin solution was filter-sterilized (0.22 µm) while the L-cysteine solution was autoclaved (121 ºC, 10 min). The reactors were inoculated with active *C. carboxidivorans* cells to achieve an initial optical density at 600nm (OD_{600}) of 0.05. Temperature was maintained at 35 ºC and agitation at 250 rpm during all the experiment.

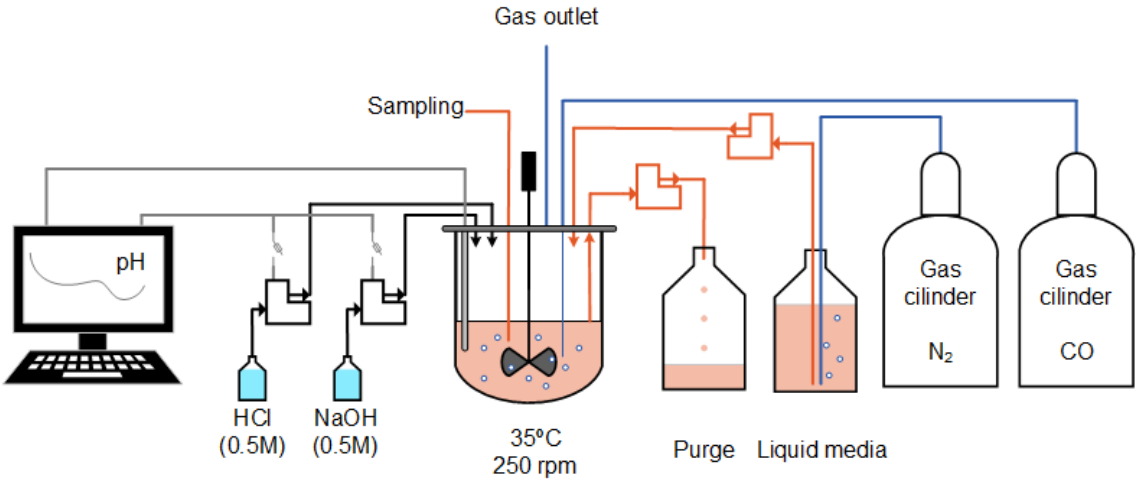

Figure 9.1. Experimental set-up for CSTR with *C. carboxidivorans*.

The pH was continuously monitored and controlled at desired set-points with NaOH (0.5 M) or HCl (0.5 M). Batch mode was operated for ~24 h, after which the pH control and the addition of the liquid feed started. Liquid medium in the feed tank was

prepared as previously described and sparged with N_2 to accomplish anaerobic conditions. The experimental plan is summarized in Table 9.1. Two different pH set-points (5.4 and 5.6) were compared to evaluate the performance of the CSTR with *C. carboxidivorans.* The tested dilution rates were of 0.034; 0.050 h^{-1} (pH = 5.4) and 0.034; 0.050; 0.083 and 0.167 h^{-1} (pH = 5.6). Residence times were ~30; 20; 12 and 6 h, respectively. Each dilution rate value was tested during a minimum of 4 residence times. Additionally, at the pH set-point of 5.4 and dilution rate of 0.05 h^{-1} a punctual addition of exogenous ethanol was introduced to the reactor in order to enhance the production and productivity of the bioalcohols.

Table 9.1. Experimental plan with the CSTR for syngas fermentation.

pH set-point	**Dilution rate (h^{-1})**			
5.4	0.034	0.050		
5.6	0.034	0.050	0.083	0.167

9.2.3. Analytical methods

Samples were taken at desired time points using sterile single-use syringes from the reactors. The OD_{600} was measured with a UV-vis spectrophotometer (SPECORD 50 UV-VIS, Analytik Jena, Jena, Germany). Biomass concentration (gDM L^{-1}) was calculated with a previously calibrated curve (1 OD_{600} = 0.4 gDM L^{-1}). Liquid samples were centrifuged at 13000 rpm for 10 min (Centrifuge MiniSpin, Eppendorf Italia, Milan, Italy) prior analysis of fermentation metabolites. Acids (acetic, butyric and hexanoic acid) and solvents (ethanol, butanol and hexanol) were detected using an HPLC (HP1100, Agilent Co., Santa Clara, CA, USA). Liquid concentrations were determined using a refractive index detector (RID) with a Rezex ROA-Organic Acid H+ (8%) column (300 x 7.8 mm, Phenomenex, Italy) operated at 60 ºC. The mobile phase was 3.5 mM of sulphuric acid with a flow of 1 mL min^{-1}. Productivity of metabolites was stablished as the product of liquid concentration by dilution rate (mg L^{-1} day^{-1}).

9.3. Results and discussion

The effect of the dilution rate and the pH was evaluated in a CSTR configuration to assemble medium carbon chain alcohols (C4-C6). Figure 9.2 and Figure 9.3 show the time course of the experiments for the reactor 1 (R1) and reactor 2 (R2), respectively. The productivities of the metabolites for both reactors are summarized in Table 9.2. At early step, the reactors were continuously fed with CO but the liquid medium was kept on batch mode to promote biomass growth. The microorganism started to grow without

a visible lag phase in any of the reactors. During the batch mode the pH was uncontrolled in both fermenters resulting in a simultaneous production of biomass and C2-C4 metabolites (Figure 9.2 and Figure 9.3). After ~24 h, the continuous mode of operation started at a dilution rate of 0.034 h^{-1} along with the pH control at 5.4 (R1) and 5.6 (R2). At 144 h, biomass reached pseudo-steady state conditions in both reactors with a concentration about 0.45 g L^{-1}. The C2 and C4 bioalcohols were produced concomitant to acid production since starting. The productivities at steady state (~200 h in both reactors) were 349 ± 24 (R1, pH = 5.4) and 235 ± 60 (R2, pH = 5.6) mg L^{-1} day^{-1} for ethanol and 145 ± 12 (R1, pH = 5.4) and 147 ± 19 (R2, pH = 5.6) mg L^{-1} day^{-1} for butanol, respectively. The C6 compounds were produced later. Hexanoic acid was first detected at about 48 for both reactors. However, in R1 (pH = 5.4), hexanol appeared at time 144 h (4 residence times equivalent) and in R2 (pH = 5.6) at 70 h (1.5 residence time equivalent). Thus, the rise in 0.2 pH units led to an earlier hexanol production. Moreover, the hexanol productivity was 3-fold higher in R2 (pH = 5.6) with 138 ± 13 mg L^{-1} day^{-1} than in R1 (pH = 5.4) with 46 ± 13 mg L^{-1} day^{-1}. Up to now, there is a lack of studies on the influence of pH for *C. carboxidivorans* in continuous operation. Fernández-Naveira et al. (2017b) have reported the production of C6 metabolites on stirred tank reactors with continuous gas-feeding. These researchers observed that a pH set-point of 5.75 required >100 h to produce C6 metabolites. Alternatively, the maintenance of the pH at 5.75 during 24 h and natural acidification to a pH of 4.75 anticipated hexanoic acid production at 68 h although hexanol did not appeared until 120 h of fermentation. Our data suggest that the assembly of C6, specially hexanol, is promoted by the combination of simultaneous introduction of gas streams along with new nutrients in the fermentation broth when a range of pH of 5.4-5.6 is employed in the HBE fermentation.

Table 9.2. Performance summary for R1 (pH 5.4) and R2 (5.6).

Dilution rate (h^{-1})	**P_{AA} (mg L^{-1} day^{-1})**	**P_{EtOH} (mg L^{-1} day^{-1})**	**P_{BA} (mg L^{-1} day^{-1})**	**P_{ButOH} (mg L^{-1} day^{-1})**	**P_{HA} (mg L^{-1} day^{-1})**	**P_{HexOH} (mg L^{-1} day^{-1})**
R1 (pH 5.4)						
0.034	510 ± 58	349 ± 24	127 ± 13	145 ± 12	73 ± 6	46 ± 13
0.050	548 ± 109	455 ± 166	149 ± 39	179 ± 37	88 ± 30	66 ± 4
R2 (pH 5.6)						
0.034	328 ± 18	235 ± 60	172 ± 10	147 ± 19	205 ± 29	138 ± 13
0.050	483 ± 65	348 ± 55	181 ± 29	175 ± 21	179 ± 25	118 ± 7
0.083	914 ± 91	490 ± 33	282 ± 10	192 ± 40	203 ± 18	100 ± 19

P_{AA}: acetic acid productivity; P_{EtOH}: ethanol productivity; P_{BA}: butyric acid productivity; P_{ButOH}: butanol productivity; P_{HA}: hexanoic acid productivity; P_{HexOH}: hexanol productivity

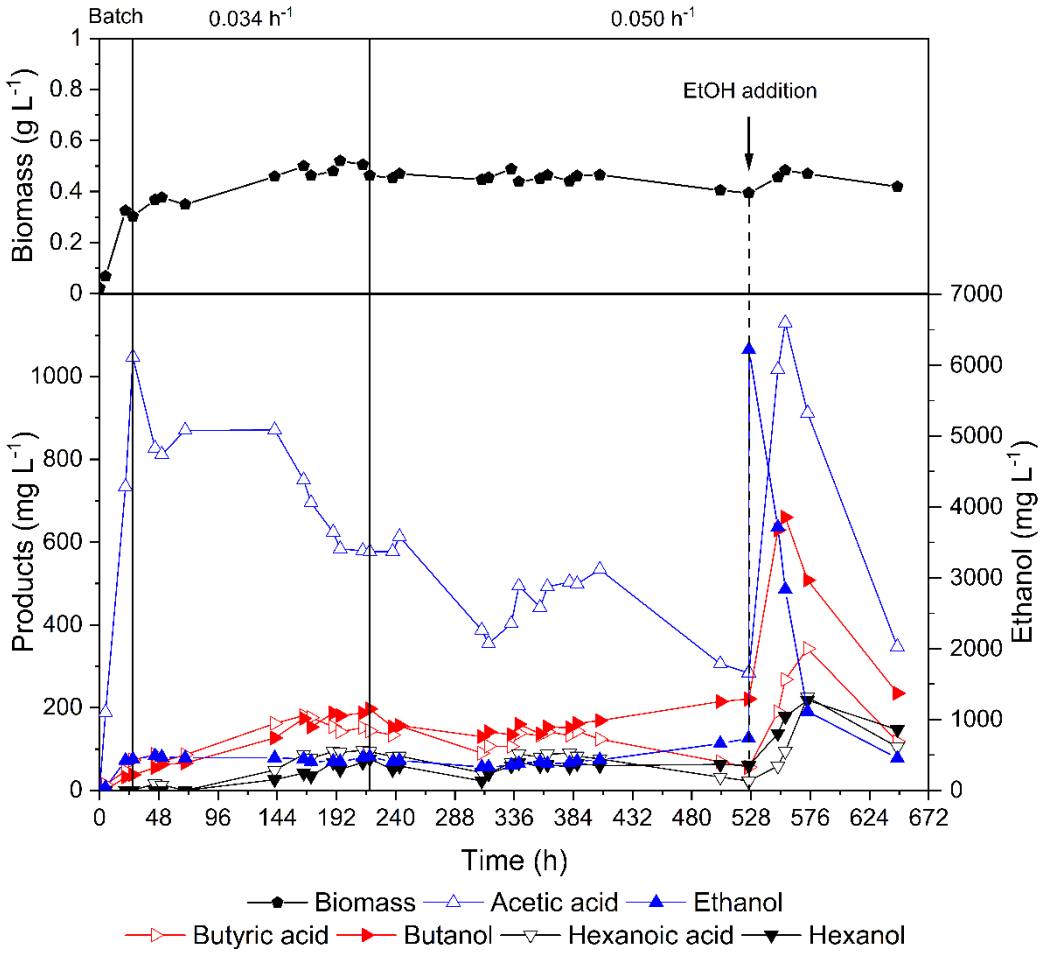

Figure 9.2. R1, gas-liquid CSTR at a pH set-point of 5.4. Production of biomass (g L^{-1}) and metabolites (mg L^{-1}). The right axis corresponds to ethanol concentration.

At ~200 h, the dilution rate was changed to 0.05 h^{-1} in both reactors. The 40% increase on the dilution rate led to lower concentrations of metabolites. Despite this, in R1 (pH = 5.4), the ethanol, butanol and hexanol productivities raised to 455 ± 166, 179 ± 37 and 66 ± 4 mg L^{-1} day^{-1}, which represents a ~1.3; ~1.2 and ~1.4-fold higher values over the previous dilution rate (0.034 h^{-1}). Meanwhile, in R2 (pH = 5.6), the ethanol and butanol productivities were 348 ± 55 and 175 ± 21, leading to a ~1.5 and ~1.2-fold higher values compared to the dilution rate of 0.034 h^{-1}. However, a 118 ± 7 mg L^{-1} day^{-1} hexanol productivity was observed which is slightly lower than the one obtained at the previous dilution rate (Table 9.2). The increase of the pH set-point from 5.4 (R1) to 5.6 (R2) seems to improve the hexanol productivities in the CSTR operations with *C. carboxidivorans*. In this regard, the hexanol productivity in the R2 (pH = 5.6) resulted in a 1.8-fold improved value in comparison with the R1 (pH = 5.4).

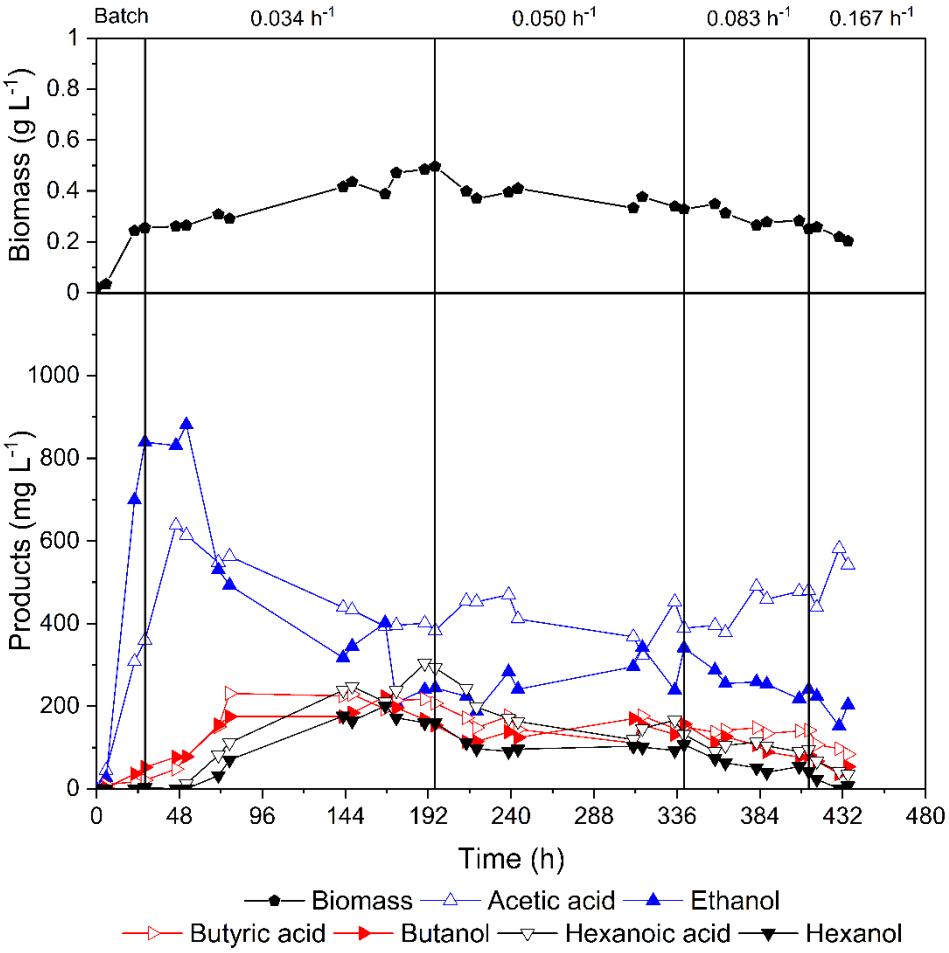

Figure 9.3. R2, gas-liquid CSTR at a pH set-point of 5.6. Production of biomass (g L^{-1}) and metabolites (mg L^{-1}).

In order to enhance the production and productivity of the R1 (pH = 5.4, dilution rate of 0.05 h^{-1}), an exogenous addition of ethanol was carried out as this alcohol may be oxidised to acetic acid by *C. carboxidivorans* (Figure 9.2, vertical arrow). After the boost of ethanol concentration at 528 h, the production of acetic acid as well as the production of the rest of C4-C6 metabolites incremented. The peak of hexanol was detected after 48 h of the ethanol addition due to the complexity of the elongation process of the carbon chain (Kottenhahn et al., 2021). Positively, the highest increment was observed on butanol and hexanol with >4.0-fold production. Thus, suggesting that the oxidation of exogenous ethanol can be used to form higher carbon compounds. Nevertheless, a sharp reduction in the concentrations of metabolites after >50 h of the ethanol addition resulted in a very similar values than the pseudo steady-state reached previously operating at such dilution rate (0.05 h^{-1}). Research focusing on oxidising ethanol to acetic acid by *Clostridium* spp. is still very scarce. A prior work reported the ability to oxidise ethanol into acetic of *Clostridium aceticum* when cultured at pH 8.0

(Arslan et al., 2019). Recently, He et al. (2022) also described that an enriched anaerobic culture with CO, in which *C. carboxidivorans* and other *Clostridium* spp. were present, was able to increment the production of acetic and butyric acids and butanol in batch mode by adding exogenous ethanol. Following with the reported data in batch modes, our results demonstrated that productivities could be improved in continuous operation with *C. carboxidivorans* by the introduction of exogenous ethanol. However, the system finally recovered a similar concentration of metabolites previous to the addition of exogenous ethanol. Thus, a fed-batch strategy adding ethanol on 1-2 days basis would promote the formation of higher carbon metabolites.

The R2 (pH = 5.6) was chosen as the best candidate for increasing the dilution rate to 0.083 h^{-1} at 340 h. At this dilution rate, a progressive decrease in biomass concentration was observed up to a value of 0.29 ± 0.04 g L^{-1}. After 6 residence times of operation (412 h), the steady-state productivities for ethanol, butanol and hexanol were 490 ± 33; 192 ± 40 and 100 ± 19 mg L^{-1} day^{-1}, respectively (Table 9.2). The productivity of the C2 bioalcohol incremented by ~1.4-fold, whereas the productivity of the C4 and C6 bioalcohols were similar to the previous dilution rate. At 412 h, the dilution rate was doubled to 0.167 h^{-1}. Under this condition biomass and metabolites sharply decreased. The greatest impact on metabolite profiles was on C6 compounds with a decrease on the hexanol concentration in the reactor to a value of ~10 mg L^{-1}. This concentration was reached in less than ~4 residence times indicating the decline of the ability to produce this bioalcohol. Meanwhile the acetic acid concentration increased from 480 to 582 mg L^{-1} before stopping the experiment. This observation was related to that the C2 acid is the first metabolite to be produced in the HBE fermentation. Thus, the maximum dilution rate in this configuration should be kept within the range 0.05-0.083 h^{-1} for obtaining hexanol productivities of approximately 100-120 mg L^{-1} day^{-1} at pH = 5.6. These data are consistent with the specific growth rate determined previously by the hosting research group in batch serum bottles (0.060 to 0.100 h^{-1}) (Lanzillo et al., 2020). Moreover, a dilution rate of 0.1 h^{-1} was found as wash out value in a CSTR set-up at a pH of 6.0 with a mixotrophic feed (gas streams and glucose) (Vees et al., 2022). Thus, the CSTR operation with *C. carboxidivorans* may restricted up to dilution rates values of <0.1 h^{-1} when employing pH control at values ≥5.6.

The hexanol productivity versus dilution rate is plotted in Figure 9.4 for the two pHs alongside with data extracted from other studies. The data herein depicted can be considered as screening information for future research. Our results indicated that an increase in 0.2 pH units displaced the maximum productivity to lower dilution rate values;

but more than doubled it. For pH 5.6 there is a relatively broad range (0.034 and 0.050 h^{-1}) for which high hexanol productivities are reached. Future fine-tune experimentation would be done for determining the maximum productivity. These preliminary data showed the importance to zoom in the influence of pH conditions. As far as we know, there is not any literature data dealing with one-stage CSTR configuration solely feeding with CO. Regarding this, two studies dealing with cascade CSTR configuration feeding with $CO:CO_2$ mixtures are available. Both assays achieved slightly lower hexanol productivity compared our CSTR configuration (Figure 9.4). Doll et al. (2018) obtained an overall productivity of 80 mg L^{-1} day^{-1} of hexanol a cascade two CSTRs in series system with the first (acidogenic) reactor at pH = 6.0 and the second (solventogenic) reactor at pH = 5.0 with an estimated overall dilution rate of 0.048 h^{-1}. While Abubackar et al. (2018) obtained an hexanol productivity of 80 mg L^{-1} day^{-1} with a dilution rate of 0.017 h^{-1}. The results obtained with the one-stage CSTR configuration will be validated with syngas mixtures.

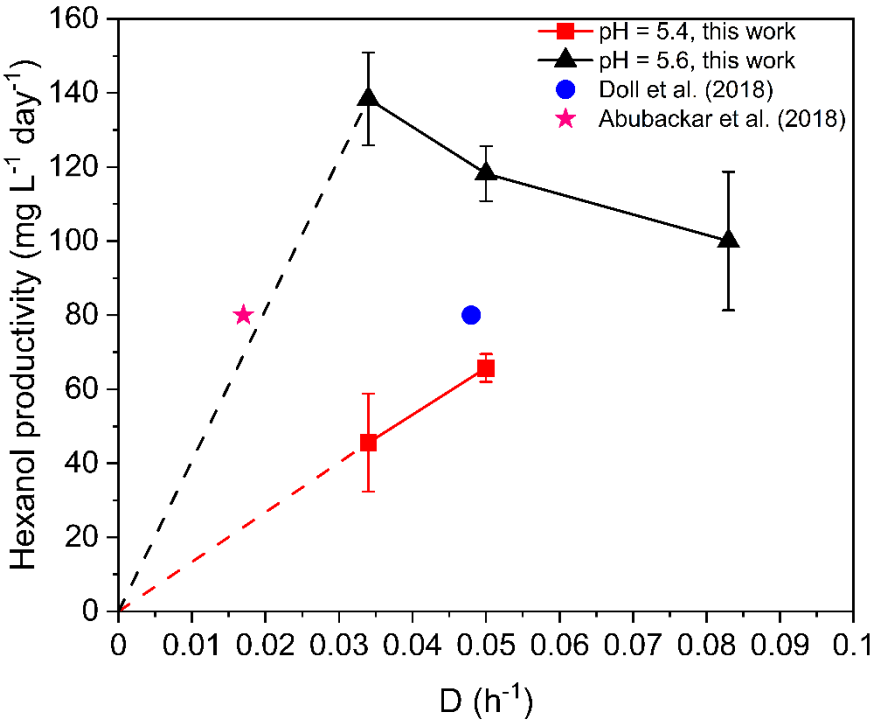

Figure 9.4. Hexanol productivity versus dilution rate obtained in the CSTR configuration with *C. carboxidivorans* versus literature data.

9.4. Conclusions

This study aimed to screen which are the operational conditions (dilution rate and pH) that promote hexanol production for *C. carboxidivorans* in a single CSTR configuration. Data showed that the use of a pH of 5.6 within the range 0.034-0.083 h^{-1} stimulated the production of C6 compounds in a single CSTR feed with pure CO. The hexanol productivity remained in relatively higher values by working at residence time ranged between 12 and 30 hours at a constant pH of 5.6 compared with literature data. Best result for hexanol productivity was 138 mg L^{-1} day^{-1} achieved with a pH of 5.6 and a dilution rate of 0.034 h^{-1}. Thus, demonstrating that the single-CSTR is a very suitable configuration to obtain hexanol with syngas produced via lignocellulosic biomass gasification such as rice straw. Future work evaluating the effect of using syngas mixtures will be carried out to foster the feasibility of this process.

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10. CONCLUSIONS AND PERSPECTIVES

The main objective of this PhD thesis was the development of fermentation models to produce bioalcohols of industrial interest by *Clostridium* spp. as a way to explore new alternatives for the exploitation of rice straw in the biorefinery context. The main conclusions from the results obtained on this work are exposed herein.

I. A recent approach to improve the economic feasibility of ABE fermentation from lignocellulosic waste lies on increasing the initial sugar concentration as a technique to improve the overall production and productivity of the process. In this work, the effect of glucose and xylose on the production of ABE solvents by *C. acetobutylicum* DSM 792 demonstrated that custom-built pH control strategies are required for avoiding acid crash phenomenon and for achieving higher butanol production and productivities.

- By selecting the conventional batch reactor configuration, the initial concentration of glucose, which represents about two thirds of the monosaccharides released from alkali pretreated rice straw, has a major impact on the pH regulation strategy for adequately transitioning from the acidogenic to the solventogenic phase. By using three different levels of glucose (33, 60, and 100 g L^{-1}), it has been shown that pH regulation should be adjusted to the expected bacterial growth to adequate balance the production and consumption rates of acetic and butyric acids. Hence, the use of low initial glucose concentration (33 g L^{-1}), for which expected maximum butanol concentration are lower than the inhibitory levels, the conventional strategy based on buffering the fermentation media is the best strategy over the online control of pH. This is due to the lack of pH control results in a slower glucose consumption at the initial stages, which is linked to a slower acid production. This is beneficial for ensuring a minimum glucose level for the maintenance during solventogenesis step. In contrast, higher monosaccharide solutions (≥60 g L^{-1}) requires the online control of the minimum pH value on a range of 4.8-5.1 to guarantee that the undissociated form of acetic and butyric acids keep below acid crash levels. This work demonstrated that following this strategy is possible to achieve high butanol titers (~11 g L^{-1}) with *C. acetobutylicum* DSM 792. If sugar concentration techniques are employed on rice straw hydrolysates, it could be processed up to 100 g L^{-1} without substrate inhibition. In this case, a novelty strategy ("*hybrid pH control*") based on the natural evolution of pH until reaching the spontaneous minimum pH (<4.6) followed by the control of the pH_{min}>5.1

allowed to achieve a butanol production of 15.80 g L^{-1}. These results are of interest for industrial scale-up as could enhance the overall productivity of conventional batch ABE fermentation when combining with ISPR techniques.

- As xylose constitutes about one third of the monomeric composition of the rice straw hydrolysate, this research focused on the efficient usage of this monosaccharide to improve the consumption of the whole carbon content of the lignocellulosic waste. The results from pure xylose fermentation media are of high interest when using xylose-rich solutions as it is the case of cascade reactors for sequential carbon uptake, as well as when fermenting xylose in presence of glucose in one-stage reactors. In this regard, this work showed that the use of pure xylose as carbon substrate modulates differently the metabolism of *C. acetobutylicum* when comparing to glucose. After 72 hours from inoculation, the production of butanol was halved (~5 g L^{-1}) when an online pH control (pH_{min}>4.8-5.1) was used in comparison with just buffering fermentation media (~8 g L^{-1}). As the biomass yield is higher employing xylose than glucose, the strategy of minimum pH control hindered the reduction potential of the media, thus declining the adequately shift from acidogenic to solventogenic stages.

- Experimental data demonstrated that pH regulation impacts in an opposite way to the usage of both monosaccharides when they coexist in sugar mixtures. The xylose uptake is hampered by the presence of glucose despite the beneficial effect of the pH regulation, hence, its conversion into biobutanol using lignocellulosic hydrolysates is low with *C. acetobutylicum*. The data collected in reactors with xylose in presence of glucose (total monosaccharide concentration of 60 g L^{-1}) indicated that higher xylose concentrations on the media are easier to be assimilated with pH_{min}>5.1 (glucose:xylose mass ratio 1:1), whereas the fermentation media controlled with pH_{min}>4.8 enhances the depletion of xylose when lower concentrations are used (glucose:xylose mass ratio 3:1). For this reason, *ad hoc* pH regulations might be developed the specific monomeric sugar composition when employing lignocellulosic hydrolysates with *C. acetobutylicum*.

II. A new proof of concept consisting in a continuous reactor for ABE fermentation with biomass retention onto plastic rings has been successfully developed. By augmenting the available microbial population, the butanol and solvent productivity of the CSTR was increased by four compared (butanol: 2.4 g L^{-1}

h^{-1}; ABE: 3.7 g L^{-1} h^{-1}) to the best results obtained in the batch fermenters (butanol: 0.6 g L^{-1} h^{-1}; ABE: 0.9 g L^{-1} h^{-1}). Despite the excellent increment in solvent productivities, the continuous addition of both monosaccharides led to poor xylose uptake due to glucose repression. Consequently, more research is required to enhance the xylose conversion.

III. The pH control strategy based on the set-point of a minimum pH developed on this PhD thesis was successfully implemented using alkali pretreated rice straw hydrolysate obtaining better solvent productions over the usage of pure monomeric sugars. Regarding this, the lack of inhibition observed by the employment of the rice straw hydrolysate demonstrated the scalability of the process.

IV. The combination of *C. acetobutylicum* and *S. cerevisiae* in a co-culture strategy showed the best usage of the rice straw hydrolysate when minimum pH is controlled at 4.8 (butanol: 7.0 g L^{-1}; ABE: 13.1 g L^{-1}). The co-culture fermentation incremented in a ~3-fold the bioethanol production compared to the *Clostridium* monoculture. Additionally, the xylose consumption was nearly complete (94%) and doubled the monosaccharide conversion when comparing with the results from the batch *Clostridium* monoculture (47%). Thus, the obtained results effectively demonstrated that *C. acetobutylicum* and *S. cerevisiae* co-culture is a feasible way to maximise the conversion of rice straw hydrolysates to produce bioalcohols. Additionally, the co-culture model developed may be transferred to other fermentations that are hindered by the pentose utilization.

V. The production of medium carbon chained bioalcohols, especially biobutanol and biohexanol, by *C. carboxidivorans* has been demonstrated employing a single CSTR with pH control and pure CO, main compound of syngas. Notably, biohexanol productivity, the most difficult metabolite to be produced by this microorganism, remained stable when operating at a dilution rate range of 0.034-0.083 h^{-1} when working at a pH set-point of 5.6. Best literature data regarding hexanol productivity was obtained (100-140 mg L^{-1} day^{-1}) under these conditions. Meanwhile, the doubling of the dilution rate to 0.167 h^{-1} resulted in very low biohexanol production. These results open the door to the gasification treatment of rice straw to produce bioalcohols from syngas via HBE fermentation.

The work of this PhD thesis has deepened on the behaviour of *Clostridium* spp. to produce bioalcohols on batch and continuous fermenters for the better exploitation of lignocellulosic feedstocks such as rice straw. The experimental results have proportioned useful information regarding the understanding of the processes when working with advanced configurations such as the co-culture of microorganisms or the continuous operation. From this PhD thesis, the following future research is proposed:

I. Optimization of the CSTR operation with *C. acetobutylicum* and other *Clostridium* spp. for the better exploitation of the xylose fraction from the carbon source. Considering the application of other support matrixes (e.g. activated carbon, exhausted rice straw, etc.) to retain active biomass.

II. Study of the immobilization of *C. acetobutylicum* and *S. cerevisiae* in order to increment ABE productivity and accelerate xylose consumption.

III. Transference of the batch fermentation model to fed-batch mode with ISPR techniques to increase solvent productivities.

IV. Evaluation of the effect using syngas mixtures on biohexanol productivity for the HBE fermentation in the configuration proposed on this PhD thesis.

11.Resumen extendido

ANTECEDENTES

Ante la grave amenaza que supone el cambio climático, los gobiernos están tomando medidas para contener las emisiones de gases de efecto invernadero. En la Unión Europea se han implementado políticas como el “Pacto Verde Europeo” que tiene como objetivo la neutralidad climática en 2050. Una de las estrategias prioritarias para alcanzar este objetivo es el viraje a hacia una Economía Circular. Esta estrategia pretende impulsar un nuevo modelo de producción y consumo basado en la minimización de los residuos mediante la reutilización, reparación y reciclado de los mismos para su posterior incorporación al sistema de producción siempre que sea posible. En este contexto, las biorefinerías basadas en el uso de residuos para la producción de bioalcoholes por via fermentativa contribuyen al objetivo de descarbonización sostenible al reducir la dependencia de las fuentes de materia prima no renovables. De entre todos los posibles residuos, los residuos lignocelulósicos destacan por su gran abundancia y bajo coste.

Los residuos lignocelulósicos presentan una estructura formada principalmente por celulosa, hemicelulosa y lignina que dificulta su utilización como materia prima en la producción de bioalcoholes por via fermentativa. Existen varias alternativas que pueden transformar estos residuos en sustratos utilizables por los microorganismos. La alternativa más empleada consiste en la liberación de los monosacáridos que componen la celulosa y la hemicelulosa. Este proceso requiere generalmente de dos etapas, un pretratamiento que aumenta la biodegradabilidad de la materia prima y una hidrólisis enzimática en la que se obtienen los monosacáridos fermentables. Entre los microorganismos que pueden utilizar esta alternativa para producir bioalcoholes se encuentran las bacterias solventogénicas del género *Clostridium*, como es *Clostridium acetobutylicum* bacteria utilizada en esta tesis doctoral. Este microorganismo es capaz de producir biobutanol como producto principal mediante la fermentación acetona-butanol-etanol (ABE). Otra alternativa que está cobrando interés es la gasificación de los residuos lignocelulósicos en gas de síntesis. Estas corrientes gaseosas pueden ser usadas por algunas bacterias del género *Clostridium* para producir bioalcoholes. Entre estas bacterias se encuentra *Clostridium carboxidivorans*, microorganismo capaz de metabolizar el gas de síntesis a biohexanol mediante la fermentación hexanol-butanol-etanol (HBE).

La selección en esta tesis doctoral de la paja de arroz como residuo lignocelulósico está motivada por su disponibilidad en el Parque Natural de l’Albufera, situado en las cercanías de la ciudad de Valencia. Este paraje natural es uno de los

humedales más importantes de la cuenca mediterránea y en el que se cultiva un 15% de la producción española de arroz. Este cultivo deja como residuo la paja de arroz en el campo tras la cosecha. Tradicionalmente, la eliminación de la paja se ha llevado a cabo mediante su quema al aire libre en el propio terreno de cultivo. Sin embargo, esta forma de gestión provoca la emisión de contaminantes atmosféricos perjudiciales que afectan al ambiente y la salud de la población además de la producción de gases de efecto invernadero. Este método está en desuso, ya que contraviene las políticas actuales de la Unión Europea. En este contexto, la búsqueda de nuevas formas de explotación de estos residuos, como es su uso para la producción de bioalcoholes, crea una nueva via para la gestión de la paja de arroz.

El trabajo de esta tesis doctoral se centra en la producción de biobutanol y biohexanol usando bacterias solventogénicas del género *Clostridium* para explorar nuevas propuestas de explotación de residuos lignocelulósicos en el marco de la Economía Circular. Para llevar a cabo este objetivo se plantearon una serie de objetivos parciales: 1) obtener un modelo de fermentación ABE para la explotación de los monosacáridos presentes en los residuos lignocelulósicos usando *C. acetobutylicum*; 2) llevar a cabo una prueba de concepto de fermentación en continuo con retención celular y control de pH para incrementar la productividad de butanol; 3) validar el modelo de fermentación en discontinuo desarrollado con azúcar modelo utilizando hidrolizado de paja de arroz; 4) evaluar el uso de un proceso de co-cultivo de *C. acetobutylicum* y *Saccharomyces cerevisiae* para incrementar la conversión del hidrolizado de paja de arroz en disolventes ABE y 5) estudiar una configuración de fermentación continua usando *C. carboxidivorans* y monóxido de carbono, principal componente del syngas, para producir bioalcoholes.

RESUMEN DE RESULTADOS

El primer trabajo de esta tesis doctoral fue publicado en la revista "*Biochemical Engineering Journal*" con el nombre de "*The combined effect on initial glucose concentration and pH control strategies for acetone-butanol-ethanol (ABE) fermentation by Clostridium acetobutylicum DSM 792*". Este estudio tuvo como objetivo la evaluación sistemática del efecto combinado de la concentración inicial de glucosa y la estrategia de regulación de pH con *C. acetobutylicum* DSM 792. Las fermentaciones se realizaron en reactores en discontinuo (V = 0.8 L) equipados con un sistema de monitorización y control de pH. Se comparó el comportamiento de *C. acetobutylicum* DSM 792 en tres niveles de concentración de glucosa (33, 66, 100 g L^{-1}) utilizando tres estrategias diferentes de regulación de pH. La primera estrategia consistió en la formulación del

medio de fermentación con un tampón de pH basado en carbonato cálcico. En la segunda, se implementó un sistema de control para restringir el valor de pH mínimo de la fermentación. Este control se llevó a cabo utilizando conjuntamente el medio de fermentación tamponado con carbonato cálcico. En una tercera aproximación, se desarrolló una estrategia de control que consistió en una fermentación con acidificación espontánea hasta alcanzar el pH mínimo de la fermentación seguido de una subida rápida del pH a 5.1 donde se aplicó el control de pH mínimo a este valor de consigna.

En este estudio se evaluó la influencia en el patrón de fermentación de tres niveles de concentración de glucosa inicial (33, 66, 100 g L^{-1}), los cuales son representativos de las concentraciones típicas en los hidrolizados lignocelulósicos. En primer lugar, se investigaron los niveles de glucosa inicial con solo el tampón carbonato cálcico (5 g L^{-1}) como amortiguador de pH. Todos los experimentos finalizaron la fermentación en un pH mínimo de 4.6 independientemente de la concentración de glucosa empleada. En el experimento con 33 g L^{-1} de glucosa inicial se observó un agotamiento de la glucosa en menos de 24 h obteniendo una producción de 3.4 (4.0) g L^{-1} de butanol (disolventes ABE) al final de la fermentación y un rendimiento de butanol (disolventes ABE) de 0.099 (0.118) g g^{-1}. Los experimentos con 66 y 100 g L^{-1} de glucosa inicial alcanzaron una producción de butanol (disolventes ABE) de 7.5 (10.5) y 8.0 (11.6) g L^{-1}, respectivamente. Estas producciones correspondieron a unos rendimientos de butanol (disolventes ABE) de 0.155 (0.217) y 0.151 (0.220) g g^{-1} para los experimentos de 66 y 100 g L^{-1} de glucosa inicial, respectivamente. En el experimento con 33 g L^{-1} se consumió totalmente la glucosa, mientras que en los experimentos con ≥66 g L^{-1} se observó una glucosa residual de al menos 20 g L^{-1}. La mejoría en la producción y rendimiento de los experimentos con concentraciones más elevadas de glucosa inicial se relacionó con la mayor disponibilidad de monosacárido en el medio de fermentación. La incapacidad de consumir completamente la glucosa se atribuyó a la combinación de un pH bajo con una elevada producción de ácidos que se refiere al fenómeno de choque ácido. En este fenómeno, una elevada concentración de las especies sin disociar de los ácidos puede llevar a la sobreacidificación del interior de la célula interrumpiendo el metabolismo microbiano.

Con el objetivo de evitar el fenómeno de choque ácido, se implementó un sistema de control para evitar que el pH de la fermentación disminuyera por debajo de un valor de consigna. Los pHs mínimos evaluados fueron 4.8, 5.0, 5.1 y 5.5 con 60 g L^{-1} de glucosa como fuente de carbono. Estos experimentos indicaron que evitar que el pH disminuyera de un valor de 4.8-5.1 facilitaba una buena transición de la fase acidogénica

a la solventogénica dando lugar a producciones de butanol de ~11 g L^{-1}. Este control de pH (pH_{min}>4.8-5.1) incrementó la producción de butanol en un 50% al compararse con el experimento tamponado solamente con carbonato cálcico. La limitación del pH mínimo resultó en un consumo total de la glucosa en todos los pHs testados. Además, la velocidad de conversión de la glucosa se aceleró de 1.90 a 3.47 g L^{-1} h^{-1} al aumentar el valor de consigna de pH de 4.8 a 5.5. El valor de control de pH mínimo de 5.1 fue seleccionado para los siguientes ensayos debido a su mayor velocidad de consumo de glucosa (~3.0 g L^{-1} h^{-1}) y a una producción de butanol parecida (~11 g L^{-1}) a los otros valores de control de pH mínimo (pH_{min}>4.8-5.1).

Una vez seleccionado el valor de consiga de pH mínimo, se evaluó el efecto combinado del control de pH mínimo a 5.1 y la concentración inicial de glucosa (33, 66 y 100 g L^{-1}). El ensayo con 33 g L^{-1} de glucosa dio lugar a concentraciones de butanol (disolventes ABE) de 1.1 (1.6) g L^{-1}. Por contra, las producciones de butanol (disolventes ABE) con 66 y 100 g L^{-1} de glucosa fueron de 11.2 (16.1) y 13.8 (19.6) g L^{-1} empleando el control de pH mínimo de 5.1. El uso de concentraciones ≥66 g L^{-1} de glucosa con esta estrategia de control de pH mínimo incrementó en ~1.5 y ~1.7 la concentración de butanol al compararlo con los experimentos equivalentes tamponados solo con carbonato cálcico. Cabe destacar que los consumos de la glucosa fueron un 42% (66 g L^{-1} de glucosa inicial) y un 59% (100 g L^{-1} de glucosa inicial) superiores respecto a las fermentaciones llevadas a cabo solamente con el tampón. La tercera estrategia de control de pH consistente en la acidificación del medio hasta alcanzar el pH mínimo de la fermentación y un posterior incremento del pH al valor de control mínimo de 5.1 solo benefició al experimento con 100 g L^{-1} de glucosa inicial resultando en una producción de butanol (disolventes ABE) de 15.8 (23.7) g L^{-1}, la máxima obtenida en este estudio. Pese a los buenos resultados obtenidos en términos de producción de solventes, esta estrategia de regulación del pH puede ser difícil de implementar. Por contra, la estrategia de control de pH mínimo es un método sencillo con el cual se obtuvieron resultados robustos para concentraciones ≥66 g L^{-1} de glucosa inicial. En base a estos resultados, la estrategia de control de pH mínimo fue la utilizada durante los siguientes estudios de esta tesis doctoral con la bacteria *C. acetobutylicum* DSM 792.

El siguiente trabajo de esta tesis fue publicado en la revista "*Fermentation*" bajo el nombre de "*The influence of sugar composition and pH regulation in batch and continuous acetone-butanol-ethanol fermentation*". Este estudio tuvo como objetivo ampliar la evaluación de las estrategias de regulación de pH desarrolladas en el anterior trabajo a xilosa y mezclas de glucosa:xilosa. En fermentaciones en discontinuo se

investigó el efecto combinado de la formulación del tampón (carbonato cálcico o acetato amónico) con el control de pH mínimo en tres composiciones de azúcares: xilosa pura y dos mezclas de glucosa:xilosa (1:1 y 3:1 con un total de 60 g L^{-1} de azúcares). En la siguiente parte del estudio y con el objetivo de incrementar la productividad de butanol respecto a la configuración en discontinuo, se llevó a cabo una prueba de concepto de un reactor en continuo de tanque agitado (V = 75 mL) con un sistema de retención de biomasa que consistió en añadir anillos plásticos. En el experimento en continuo se empleó un protocolo de monitorización de pH idéntico a la configuración en discontinuo. Para la puesta en marcha se utilizó una corriente de alimento con 60 g L^{-1} de glucosa y una estrategia de control de pH en dos niveles: en primer lugar, un valor de consigna de pH mínimo de 6.0 para favorecer la acidogenia y el crecimiento de la biomasa. Durante esta etapa se evaluaron factores de dilución de 0.042 a 0.167 h^{-1}. Una vez desarrollada la biomasa, el pH disminuyó hasta un valor de 4.8 para estimular la solventogenia. La producción de solventes fue evaluada empleando los factores de dilución de 0.167 y 0.333 h^{-1}. Además, durante este último factor de dilución se investigó el funcionamiento del reactor con un alimento compuesto por una mezcla de glucosa:xilosa (40:20 g L^{-1}).

La utilización de xilosa (60 g L^{-1}) como única fuente de carbono dio lugar a patrones de fermentación diferentes a los obtenidos con glucosa pura en el anterior trabajo. Los resultados obtenidos mostraron que el control de pH mínimo incrementó la biomasa producida en aproximadamente ~1.7 (acetato amónico, pH_{min}>4.8) y ~1.4 veces (carbonato cálcico, pH_{min}>5.1) al compararlo con los experimentos con su correspondiente tampón sin control de pH mínimo. Las fermentaciones sin control de pH mínimo llegaron a concentraciones de butanol de ~8.0 g L^{-1} independientemente del tampón utilizado. Por el contrario, la estrategia de control de pH mínimo afectó negativamente a la producción de solventes al observarse una disminución de la producción de butanol de ~8.0 a ~5.5 g L^{-1} con cualquiera de los dos tampones empleados (carbonato cálcico y acetato amónico). Además, independientemente de la regulación de pH (formulación de tampón y/o limitación del pH mínimo) no se utilizó toda la xilosa, alcanzándose consumos similares. La presencia de xilosa residual en el medio de fermentación se relacionó con la inhibición por producto, ya que una concentración de 4.5 g L^{-1} de butanol reduce el consumo de xilosa en un 50% y una concentración de ~8.0-8.5 g L^{-1} es inhibitoria para *C. acetobutylicum* con este monosacárido. Los resultados indicaron claramente que *C. acetobutylicum* se comporta de forma diferente cuando es cultivada con únicamente glucosa o xilosa, lo que demostró la importancia de evaluar la utilización de las mezclas de glucosa y xilosa como fuente de carbono.

Para observar el efecto de la xilosa en presencia de glucosa se evaluaron dos ratios diferentes de glucosa:xilosa (1:1 y 3:1) con *C. acetobutylicum*. La primera ratio ensayada fue la 1:1 (60 g L^{-1} de azúcares totales). De las estrategias de regulación de pH evaluadas (formulación de tampón y control de pH mínimo), el ensayo con carbonato cálcico y pH_{min}>5.1 mostró un crecimiento diaúxico ligado al consumo de xilosa. El metabolismo de xilosa empezó tras 24 h de la finalización de la glucosa disponible produciendo simultáneamente butanol hasta una concentración de 7.2 g L^{-1}. Por el contrario, en el ensayo llevado a cabo con el tampón acetato amónico y el control de pH (pH_{min}>4.8) se observó un consumo bajo de xilosa sin asociarse a un aumento de la concentración de butanol. En la siguiente ratio evaluada se disminuyó la proporción de xilosa (ratio 3:1, 60 g L^{-1} de azúcares totales) para analizar unas condiciones más similares a las encontradas en los hidrolizados lignocelulósicos, donde la xilosa se encuentra normalmente a menor concentración respecto la glucosa. En esta ratio, el control de pH (pH_{min}>4.8-5.1) junto al carbonato cálcico no favoreció el metabolismo de xilosa después del agotamiento de la glucosa al contrario de lo observado con una mayor concentración de xilosa (ratio 1:1). Los resultados obtenidos indicaron que cuando se utiliza carbonato cálcico como especie tamponante es necesaria una concentración mínima de xilosa para favorecer las rutas de consumo de este monosacárido en *C. acetobutylicum*. Por otra parte, el experimento en el que se observó una mayor conversión de xilosa con la ratio 3:1 fue el tamponado con acetato amónico y control de pH (pH_{min}>4.8) alcanzando una producción de butanol (disolventes ABE) de 8.8 (13.2) g L^{-1} asociada a una productividad máxima de butanol (disolventes ABE) de 0.61 (0.89) g L^{-1} h^{-1}. En base a estos resultados, se seleccionó el tampón acetato amónico y la estrategia de control de pH mínimo para llevar a cabo la prueba de concepto de reactor en continuo.

En el reactor en continuo la producción de biomasa se incentivó al implementar un control de pH mínimo a un valor de consigna de 6.0 desde el inicio del experimento. La operación en continuo comenzó tras 30 h de funcionamiento en discontinuo con una corriente de alimento de 60 g L^{-1} de glucosa. El factor de dilución fue duplicándose cada 48 h desde un valor de 0.042 a 0.167 h^{-1} hasta las 120 h. Durante esta etapa la biomasa se retuvo de forma exitosa sobre el soporte inerte. A las 144 h de fermentación (factor de dilución de 0.167 h^{-1}), la disminución del valor de consigna de pH de acidogenia (pH_{min}>6.0) al valor de solventogenia (pH_{min}>4.8) resultó en una producción de butanol (disolventes ABE) de 9.0 (10.1) g L^{-1} lo que indicó una buena transición a la fase solventogénica por parte de *C. acetobutylicum*. A continuación (192 h), el factor de dilución fue duplicado a un valor de 0.333 h^{-1}, alcanzándose una concentración en

estado estacionario de butanol (disolventes ABE) de 6.8 (11.1) g L^{-1} correspondiente a una productividad de 2.3 (3.7) g L^{-1} h^{-1} con 60 g L^{-1} de glucosa. El cambio de glucosa pura a una alimentación con una mezcla de glucosa y xilosa (ratio 2:1) resultó en una concentración en estado estacionario de butanol (disolventes ABE) de 7.1 (11.2) g L^{-1} equivalente a una productividad de 2.4 (3.7) g L^{-1} h^{-1}, la máxima alcanzada en este trabajo, cuadruplicando la productividad obtenida en la configuración en discontinuo en condiciones similares. No obstante, durante esta fase el consumo de xilosa fue menor que en los reactores en discontinuo (18% versus 74%) lo que se relacionó con la disponibilidad constante de glucosa en el reactor al acentuarse la represión por catabolito de carbono.

El siguiente trabajo de esta tesis fue publicado en la revista "*Biomass conversión and Biorefinery*" bajo el nombre de "*Solvent production from rice straw by a co-culture of Clostridium acetobutylicum and Saccharomyces cerevisiae: effect of pH control*". El principal objetivo de este estudio fue la evaluación de un sistema en co-cultivo con *C. acetobutyicum* y *S. cerevisiae* para mejorar la producción de disolventes y la conversión de monosacáridos presentes en el hidrolizado de paja de arroz en modo discontinuo. Para lograr este objetivo se diseñó el siguiente plan experimental. Inicialmente, se evaluó el efecto de la regulación del pH en sustratos modelo. A continuación, con la estrategia de control de pH (acetato amónico y pH_{min}>4.8) se validaron los resultados previos empleando hidrolizado de paja de arroz pretratada con hidróxido sódico. En la siguiente parte de este estudio se evaluó un sistema de co-cultivo en el que inicialmente se determinó el tiempo de inoculación de los dos microorganismos en un experimento de pre-barrido en botellas de suero de 50 mL. El primer microorganismo inoculado fue *C. acetobutylicum* y posteriormente se introdujo *S. cerevisiae* con 0, 5 y 10 h de desfase utilizando una mezcla sintética de glucosa y xilosa que mimetizaba la concentración del hidrolizado de paja de arroz (glucosa: 35 g L^{-1} y xilosa: 15 g L^{-1}). A continuación, se llevaron a cabo experimentos de co-cultivo utilizando reactores en discontinuo con el sistema de monitorización y control de pH mínimo desarrollados en esta tesis doctoral. Para ello se utilizó el tampón acetato sódico y el control de pH mínimo (pH_{min}>4.8) con dos fuentes de carbono, el medio sintético mimetizando el hidrolizado (glucosa: 35 g L^{-1} y xilosa: 15 g L^{-1}) y el hidrolizado de paja de arroz (glucosa: 34 g L^{-1} y xilosa: 13 g L^{-1}).

El uso de acetato amónico como agente tamponante resultó en una producción de ~11 g L^{-1} de butanol independientemente de desarrollar las fermentaciones con acidificación espontánea o con un control de pH mínimo, al contrario que lo observado con el uso de carbonato como amortiguador de pH. No obstante, sí que se obtuvieron

diferencias significativas en la velocidad de consumo de glucosa acelerándose en ~2.8 veces (0.96 a 2.67g L^{-1} h^{-1}) al utilizar el control de pH mínimo (pH_{min}>4.8). Por este motivo, se seleccionó la estrategia de control de pH mínimo (pH_{min}>4.8) con el tampón acetato para llevar a cabo la fermentación del hidrolizado de paja de arroz. Este ensayo resultó en una concentración de butanol (disolventes ABE) de 6.5 (9.5) g L^{-1} con un consumo total de la glucosa y de un 47% de la xilosa disponible en el hidrolizado de paja de arroz (glucosa: 34 g L^{-1} y xilosa: 13 g L^{-1}). Las velocidades de consumo de glucosa fueron 0.96 g L^{-1} h^{-1} (tamponado solamente con acetato amónico) y 2.38 g L^{-1} h^{-1} (pH_{min}>4.8). Estas velocidades de consumo fueron similares a los valores obtenidos con sustratos modelos indicando que no hay inhibición en el consumo de glucosa cuando se utilizada hidrolizado de paja de arroz. Los resultados obtenidos al utilizar hidrolizado de paja de arroz validaron los experimentos de este estudio con sustratos modelo y control de pH mínimo para la producción de biobutanol y disolventes ABE con *C. acetobutylicum*.

En la siguiente fase de este estudio se desarrolló un sistema en co-cultivo con *C. acetobutylicum* y *S. cerevisiae* para mejorar la conversión de los monosacáridos presentes en el hidrolizado de paja de arroz a bioalcoholes. Tras un experimento de cribado se seleccionó un tiempo de inoculación de 5 h de desfase entre *C. acetobutylicum* y *S. cerevisiae* para incentivar la producción de butanol sobre etanol. A continuación, se realizaron los experimentos con mezclas sintéticas de glucosa y xilosa en concentraciones de 35 y 15 g L^{-1} y posteriormente con hidrolizado de paja de arroz. El co-cultivo con sustrato modelo y control de pH mínimo (pH_{min}>4.8) resultó en una producción de butanol (disolventes ABE) de 6.2 (10.9) g L^{-1}. Esta producción total de disolventes ocurrió en dos fases diferenciadas relativas al consumo secuencial de glucosa y xilosa. La producción de solventes durante la conversión de xilosa correspondió a 2.4 g L^{-1} de butanol y 0.8 g L^{-1} de acetona observándose un consumo de xilosa de un 85% asociado a *C. acetobutylicum*.

La fermentación del hidrolizado de paja de arroz en el co-cultivo resultó en una producción total de 7.0 (13.1) g L^{-1} de butanol (disolventes ABE), siendo el experimento con mejor producción de bioalcoholes respecto las fermentaciones en monocultivo o co-cultivo con una concentración de monosacáridos similar (glucosa: ~35 y xilosa: ~15 g L^{-1}). La utilización del hidrolizado de paja de arroz en los experimentos de co-cultivo permitió aliviar parcialmente la represión por catabolito de carbono, lo que resultó en una activación más rápida de las rutas de consumo de xilosa de 72 h respecto a las 120 h necesarias con sustrato modelo. Además, la producción de disolventes ABE utilizando

hidrolizado aumentó de 10.9 a 13.1 g L^{-1} al comparar el sistema de co-cultivo con sustrato modelo. La mejoría en el consumo de monosacáridos y producción de solventes se puede relacionar con la compleja composición de los hidrolizados de residuos lignocelulósicos. Por otro lado, el efecto del co-cultivo respecto al monocultivo de *C. acetobutylicum* con hidrolizado de paja de arroz se vio reflejado en el aumento de la producción total de disolventes ABE de 9.5 a 13.1 g L^{-1}, debido principalmente a que la concentración de bioetanol se triplicó. Es especialmente relevante el aumento del consumo de xilosa al 94%, máximo en esta tesis doctoral, al utilizar el hidrolizado de paja arroz que llevó asociado un aumento de la producción de disolventes ABE de 4.4 g L^{-1} (butanol: 2.7; acetona: 1.3 y etanol: 0.4 g L^{-1}). Estos resultados demostraron que el desarrollo del co-cultivo de *C. acetobutylicum* y *S. cerevisiae* permite prácticamente agotar los monosacáridos disponibles de los hidrolizados lignocelulósicos.

El último trabajo presentado en esta tesis doctoral se llevó a cabo durante la realización de una estancia internacional en la *Università degli Studi di Napoli Federico II* bajo la supervisión de la doctora Francesca Raganati. Este capítulo se ha denominado "*Development of a single continuous stirred tank reactor configuration with Clostridium carboxidivorans for syngas fermentation*" y parte de estos resultados se encuentran incluidos en un artículo científico que actualmente está en fase de preparación. El objetivo de este trabajo fue el desarrollo de una configuración de reactor continuo de tanque agitado alimentado continuamente con monóxido de carbono puro utilizando *C. carboxidivorans* DSM 15243 para producir bioalcoholes. En este trabajo se compararon dos valores de consigna de pH: 5.4 y 5.6. Los factores de dilución evaluados fueron 0.034; 0.050 h^{-1} (pH = 5.4) y 0.034; 0.050; 0.083 y 0.167 h^{-1} (pH = 5.6). Adicionalmente, utilizando el valor de consigna de pH de 5.4 y el factor de dilución de 0.05 h^{-1} se evaluó la adición puntual de etanol exógeno para incrementar la producción y productividad de los bioalcoholes.

Los resultados obtenidos mostraron que el reactor operado a pH 5.6 presentaba una productividad de hexanol 1.8 veces mayor que el reactor controlado a pH 5.4 con factores de dilución hasta 0.050 h^{-1}. Para aumentar la productividad de los metabolitos en el reactor a pH de 5.4 (factor de dilución de 0.050 h^{-1}) se introdujo una dosis de etanol exógena ya que podría ser oxidado a ácido acético y ser usado para producir compuestos de cadena más larga. Esta adición externa de etanol permitió cuadruplicar la concentración de butanol y hexanol en el reactor. Sin embargo, tras más de 50 h la concentración de estos metabolitos se redujo a valores previos a la adición de etanol. Por otro lado, en el reactor operado a pH 5.6 se aumentó el factor de dilución de 0.050 h^{-1}

a 0.083 h^{-1} incrementándose la productividad de etanol 1.4 veces y manteniéndose constante las productividades de butanol y hexanol. La posterior duplicación del factor de dilución a un valor de 0.167 h^{-1} provocó una disminución drástica de la capacidad de producir hexanol de *C. carboxidivorans*. Es significativo que la utilización de factores de dilución entre 0.034 y 0.083 h^{-1} resultaron en una productividad de hexanol estable comprendida entre 100 y 140 mg L^{-1} dia^{-1}, encontrándose entre los valores más elevados de la bibliografía en configuraciones de reactores continuos de tanque agitado.

CONCLUSIONES

Finalmente se presentan las principales conclusiones obtenidas en esta tesis doctoral:

I. Se ha demostrado que estrategias *ad hoc* de control de pH son necesarias para evitar el fenómeno de choque ácido y alcanzar mayores producciones y productividades de butanol y disolventes ABE mediante *C. acetobutylicum* DSM 792 con glucosa y/o xilosa.

II. Se ha desarrollado con éxito una prueba de concepto de un nuevo reactor en continuo acoplado a un sistema de retención de biomasa en anillos plásticos. El incremento de la población microbiana ha permitido cuadruplicar la productividad de butanol respecto a la máxima obtenida en un reactor en discontinuo. A pesar de la excelente mejoría en la productividad de los solventes, la constante adición de la glucosa y xilosa produjo una baja conversión de xilosa debido al efecto de represión de catabolito de glucosa.

III. Se ha aplicado con éxito una estrategia de control de pH basada en la limitación del pH mínimo de la fermentación en discontinuo de hidrolizado de paja de arroz pretratada alcalinamente. Se obtuvo mejor la producción de solventes al compararlo con azúcares modelo. En este sentido, la ausencia de inhibición observada al emplear el hidrolizado de paja de arroz demostró la escalabilidad del proceso.

IV. Se ha demostrado que la combinación de *C. acetobutylicum* y *S. cerevisiae* en una estrategia de co-cultivo permite un mejor aprovechamiento de los monosacáridos contenidos en el hidrolizado de paja de arroz. Con esta estrategia se triplicó la producción de bioetanol y duplicó el consumo de xilosa hasta consumirla casi completamente

(94%). Además, este modelo podría ser trasladado a otras fermentaciones que se vean limitadas por la utilización de pentosas.

V. Se ha demostrado que el uso de un reactor en continuo de tanque agitado con control de pH alimentado con una corriente gaseosa de CO puro, principal componente del gas de síntesis, permite la obtención de alcoholes de cadena media como butanol y hexanol. En particular, la productividad de hexanol, el bioalcohol más complicado de obtener, se mantuvo constante en el rango de factores de dilución de 0.034 a 0.083 h^{-1} empleando un valor de consigna de pH de 5.6. Los datos obtenidos bajo estas condiciones se encuentran entre los mejores de la bibliografía (100-140 mg L^{-1} dia^{-1}).

APPENDIXES

APPENDIX 1. ABBREVIATIONS

5-HMF	5-(hydroxymethyl)furfural
ABE	Acetone-Butanol-Ethanol
AMPTS II	Automatic Methane Potential Test System
CBP	Consolidated Bioprocessing
CSTR	Continuous stirred tank reactor
D	Dilution rate
DAD	Diode Array Detector
DM	Dry mass
DSMZ	*Deutsche Sammlung von Mikroorganismen und Zellkulturen*
EMP	Embden-Meyerhof-Parnas pathway
FAO	Food and Agriculture Organization
FID	Flame Ionization Detector
FPU	Filter Paper Units
GC	Gas Chromatography
GHG	Greenhouse Gases
HBE	Hexanol-Butanol-Ethanol
HPLC	High Performance Liquid Chromatography
IC	Ionic Chromatography
IEA	International Energy Agency
IPCC	Intergovernmental Panel on Climate Change
ISPR	*In situ* Product Recovery
OD	Optical Density
PKP	Phosphoketolase Pathway
PPP	Pentose Phosphate Pathway
RCM	Reinforced Clostridial Medium
RID	Refractive Index Detector
rpm	Revolutions per minute
RS	Rice Straw
USA	United States of America
UV	Ultraviolet
WCAB	Wilkins Chalgren Anaerobic Broth
YPD	Yeast-Peptone-Dextrose

APPENDIX 2. PUBLISHED PAPERS

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The combined effect on initial glucose concentration and pH control strategies for acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* DSM 792

M. Capilla, P. San-Valero, M. Izquierdo, J.M. Penya-roja, C. Gabaldón *

Research Group in Environmental Engineering (GI²AM), Department of Chemical Engineering, University of Valencia, Av. De la Universitat S/N, 46100, Burjassot, Spain

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ABSTRACT

The use and depletion of fossil fuels raised the interest in biofuels like biobutanol. *Clostridium acetobutylicum* DSM 792 is capable of producing biobutanol through ABE fermentation. Butanol production can be influenced by low sugar concentrations, like those obtained after hydrolysis of pre-treated lignocellulosic biomass. This study aimed to evaluate the influence of the initial glucose concentrations (33, 66 and 100 g L^{-1}) and pH control strategies on biobutanol production and glucose consumption. Uncontrolled pH fermentation exhibited low butanol production due to either glucose exhaustion (33 g L^{-1}) or the phenomenon of acid crash (66 and 100 g L^{-1}), which was alleviated by the use of any of the minimum pH set-points (4.8; 5.0; 5.1 and 5.5). Fermentation at a pH_{min} of 5.1 gave the best performance in butanol production and glucose consumption rate. Fermenting with a pH_{min} of 5.1 with 33 g L^{-1} of initial glucose caused acidogenic fermentation, while the use of 66 and 100 g L^{-1} of glucose led to a ~1.5 and ~1.7-fold increase in butanol concentration over their counterparts without pH control respectively. By controlling the pH after the acidogenic phase 15.8 g L^{-1} of butanol was obtained with 100 g L^{-1} of glucose, which was ~2-fold higher than without pH control. Different initial glucose concentrations therefore require different pH strategies to optimize butanol production.

1. Introduction

The extensive use of fossil fuels has led to a series of problems, such as climate change, an energy crisis and health problems. The importance of these issues has revived the interest of governments in the production of greener renewable energy. Biofuels generated biologically will become viable alternatives to oil and other derivate products when the price of biofuels becomes competitive [1]. Several types of biofuel have recently gained interest, including biomethanol, bioethanol and biobutanol. Of these, biobutanol appears especially likely to become widely used due to its physicochemical properties [2]. In addition, biobutanol has a higher heating value and lower vapour pressure than bioethanol and can be transported and distributed via existing pipelines and service stations reducing the investment cost in infrastructures [3,4].

Solventogenic *Clostridium* strains can produce biobutanol by Acetone-Butanol-Ethanol (ABE) fermentation. These *Clostridium* strains (*Clostridium acetobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium beijerinckii* or *Clostridium sporogenes*) metabolize monosaccharides (pentoses and hexoses) from different sources, such as starch or lignocellulosic biomass to produce ABE [5,6]. Among the raw materials currently available for ABE production, lignocellulosic biomass appears as an ideal candidate in terms of the circular economy, as it can be obtained from a wide range of residues such as rice straw, sweet sorghum stalk or sago hampas. Unlike the first generation of biofuels obtained from feedstocks (sugarcane, starch, molasses, etc.), lignocellulosic biomass does not interfere with the food chain supply [7].

Lignocellulosic biomass is composed mainly of cellulose, hemicellulose and lignin. However, it requires a pre-treatment and enzymatic hydrolysis to release the fermentable sugars prior to fermentation, which affects the final composition. Researchers have recently applied different pre-treatments (acid, alkali, ammonium sulphide, organic solvents or ionic liquids) to diverse lignocellulosic materials (softwood, apple pomace or rice and wheat straw) resulting in a range of 22–52 g L^{-1} of total sugars after hydrolysis depending on their conditions [5,8–11]. Unfortunately, low sugar concentrations can result in low butanol production, such as ~2 g L^{-1} of butanol with 20 g L^{-1} of glucose [12]. Novel strategies have been developed to concentrate the lignocellulosic hydrolysate, including evaporation or vacuum. However,

* Corresponding author.
E-mail address: carmen.gabaldon@uv.es (C. Gabaldón).

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these approximations increase the pretreatment cost [13,14].

ABE fermentation is characterised by two phases: an acidogenic phase followed by a solventogenic phase. In the acidogenic phase, biomass, acids (butyric and acetic) and gases (CO_2 and H_2) are produced, accompanied by a drop in pH. During the solventogenic phase, the acids are re-assimilated producing butanol, acetone and ethanol with pH recovery [15]. The bacteria selected and the external conditions, such as medium composition, temperature and pH, regulate the end products obtained [16,17]. *C.acetobutylicum* is a widely-known bacterium used for ABE fermentation [18–20], although there are still some issues that should be evaluated for efficient production of solvents [21]. In this regard, proper pH control is a key factor in ABE fermentation. High pH in the medium is related to more biomass and acids concentration in the reactors [22,23], while the reduced pH due to acid production is needed to produce solvents [24]. In fact, a minimum undissociated butyric acid concentration is necessary to initiate solvent production [25]. Moreover, the relation of the dissociated and undissociated species of acids, which are directly linked to the pH, plays an important role in the fermentation process [23]. Excessive acid production and low pH can create the conditions to cause acid crash as the undissociated acid concentration rises. As a consequence, acid crash causes the cease of glucose consumption and butanol production resulting in an ABE production failure [26].

Chemical control is now being applied in ABE fermentation, especially in continuous reactors, as a way of regulating the medium pH [27]. The pH regulation allows the study of the effect of the substrate on the butanol production. For example, butanol production was ~2.3-fold higher when xylose was used instead of lactose by keeping the pH controlled at 4.7 under continuous ABE fermentation with *C. acetobutylicum* DSM 792 [28,29]. In batch mode, Guo et al. [30] demonstrated that the best pH set-point range was between 4.5 and 4.9 when fermenting 60 g L^{-1} of glucose and *C. acetobutylicum* XY16. Alternatively, by implementing pH control exclusively during the acidogenic phase Luo et al. [31] obtained ~8.1-fold higher butanol production by avoiding acid crash with 60 g L^{-1} of glucose and *C. acetobutylicum* ATCC 824. With this pH control strategy these authors reported an increase in butanol production from 9.42 to 11.52 g L^{-1} by adding trace concentrations of inhibitors such as the phenolic compounds. Implementing of a pH control strategy would thus be beneficial for improving ABE productivity from lignocellulosic hydrolysates. Despite this, more systematic studies are required on ABE fermentation to evaluate the combined effect of the initial substrate concentration and the pH control strategy.

The aim of this study was thus to systematically evaluate the combined effect of the initial sugar concentration and the pH control strategy in ABE fermentation by *C. acetobutylicum* DSM 792 using batch reactors and glucose as the model substrate. For this, we compared the performance of the process with three different levels of initial glucose concentration (33, 66 and 100 g L^{-1}) either without pH control or with two pH control strategies: firstly, by limiting the minimum pH over the fermentation time course, and secondly by spontaneous acidogenesis without pH control followed by limiting the minimum pH during solventogenesis.

2. Material and methods

2.1. Microorganism and chemical reagents

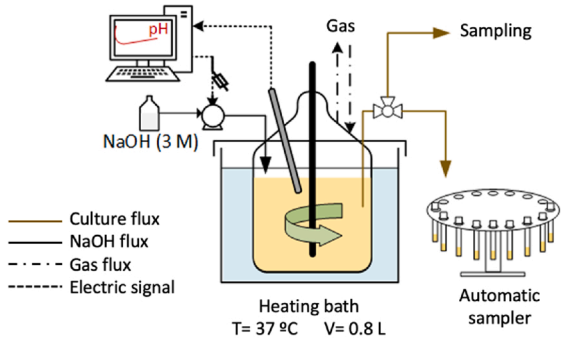
C. acetobutylicum DSM 792 was obtained from DSMZ, Germany (German Collection of Microorganisms and Cell Cultures). The cultures were maintained in 20 % glycerol at -80 °C. Prior to the experiments, the stock was cultured statically at 37 °C in 19 g L^{-1} of Reinforced Clostridial Medium (RCM) supplemented with 10 g L^{-1} of glucose as a seed inoculum. Chemicals were purchased from VWR, except for antifoam 204 (Sigma-Aldrich), $CaCO_3$ (Merk) and yeast extract (Alfa Aesar).

2.2. Experimental set-up

The fermentations were carried out in a modified 1 L bottle (total volume: 1.1 L) with a working volume of 0.8 L (stirred tank reactor, STR). The medium contained (g L^{-1}): glucose, 60 (Solution I); Yeast Extract, 5; NH_4Cl, 2; KH_2PO_4, 0.5; K_2HPO_4, 0.5; Antifoam 204, 0.01 %; resazurin sodium salt, 0.001 (Solution II); $MgSO_4{\cdot}7H_2O$, 0.2; $MnSO_4{\cdot}7H_2O$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.05 (Solution III) and $CaCO_3$, 5. All the components were sterilized prior to inoculation. Solutions I and II were autoclaved at 121 °C for 10 and 20 min, respectively. Solution III was filter-sterilized by 0.22 µm. $CaCO_3$ was autoclaved (121 °C, 20 min) inside the reactor without any liquid. The media was flushed with nitrogen for 30−35 min before inoculation to create anaerobiosis. The fermentation was carried out at 37 °C and 120 rpm with an inoculum size of 5%. The fermentations were monitored by a Tris-compatible flat pH sensor with LoggerPro software (Vernier, USA). When needed, the pH was controlled with NaOH (3 M). The pH probes were sterilized with 50 % ethanol solution for at least 12 h. Fig. 1 shows the experimental setup for the fermentation. Liquid samples from the fermentations were taken at appropriate time-points for the analysis of cell growth, glucose and products (butyric acid, acetic acid, butanol, acetone and ethanol).

2.3. Experimental plan

Four sets (runs) of experiments were planned (Fig. 1) to study the behavior of *C. acetobutylicum*. Run 1 was buffered media with 5 g L^{-1} of $CaCO_3$ without active pH control. The effect of three different initial glucose concentrations (33, 66 and 100 g L^{-1}) was evaluated in terms of butanol production and yield. A pH control strategy was applied in the following runs (run 2, 3 and 4). The pH control strategy for run 2 and run 3 consisted of natural acidification by acid production until a minimum pH set-point was reached, after which the pH was not allowed to fall by automatically adding NaOH solution. The base dosing pump was activated as many times as needed by the pH probe readings until the pH value increased 0.1 of the set-point. The spontaneous rise of pH was not controlled, allowing spontaneous pH recovery as the solventogenesis stage advanced. Run 2 was performed as the screening of several minimum pHs (4.8, 5.0, 5.1 and 5.5) to find the best pH for butanol production and glucose consumption with the intermediate initial glucose concentration (66 g L^{-1}). From these results, a pH of 5.1 was selected to



Run	glucose, g L^{-1}	pH control	set-point
1	33, 66, 100	No	
2	66	Yes	pH ≥ 4.8, 5.0, 5.1, 5.5
3	33, 66, 100	Yes	pH ≥ 5.1
4	33, 66, 100	Yes	pH ≥ 5.1 from ~24 h

Fig. 1. Experimental setup and plan for ABE fermentations.

evaluate the ABE fermentation profiles of the three initial glucose concentrations (33, 66 and 100 g L^{-1}) in run 3. A hybrid pH strategy with the three levels of initial glucose concentrations was then carried out in run 4, which consisted of natural acidification up to the spontaneous minimum pH from the acidogenesis stage, followed by a sudden increase of pH to the set-point (5.1). The pH set-point was kept as the minimum value by pH control until the end of the fermentation.

2.4. Analytical methods

Biomass concentration (gDM L^{-1}) was calculated from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany) as gDM $L^{-1} = 0.2949 \cdot OD_{600} + 0.0596$ (n = 8, $R^2 = 0.995$). The liquid samples were centrifuged at 10000 rpm for 5 min and filtered by 0.22 μm for glucose and products analysis. Glucose was analysed using an ionic chromatograph (Basic IC Plus 833, Metrohm, Switzerland) with an amperometric detector (945 Professional Detector Vario IC Amperometric Detector, Metrohm, Switzerland). The eluent was 100 mM NaOH and 3 mM sodium acetate with a flow of 0.5 mL min^{-1} and the temperature detector was set at 35 °C. Fermentation products were analysed by a gas chromatograph (7890A GC-System, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and an Rtx-VMS column (Restek, USA), using helium as the carrier gas with a flow rate of 1.1 mL min^{-1}. The oven programme was: 100 °C for 7 min; 30 °C/min until 210 and 210 °C for 5 min. The temperatures of the injector and the detector were set at 190 °C and 240 °C, respectively. Cell growth was measured by an UV-Vis spectrophotometer (SpectroFlex 6600, WTW) at 600 nm. Undissociated acid concentration was calculated by the Henderson-Hasselbalch Equation and the pKa of acetic acid (4.76) and butyric acid (4.82).

2.5. Determination of the glucose consumption rate and the specific growth rate

Glucose consumption and maximum growth rates were calculated during the exponential growth phase by Eqs. (1) and (2):

$$-q_{glucose} = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \quad (1)$$

$$\mu_{max} = \frac{\ln(X_{t2}) - \ln(X_{t1})}{t_2 - t_1} \quad (2)$$

where $-q_{glucose}$ is the glucose consumption rate (g L^{-1} h^{-1}), S_{t1} and S_{t2} are the glucose concentrations at the beginning and end of the exponential growth phase (g L^{-1}), t_2 and t_1 are the initial and end time of the exponential growth phase (h), μ_{max} is the specific growth rate (h^{-1}), X_{t1} and X_{t2} are the biomass concentrations at the beginning and end of the exponential growth (gDM L^{-1}).

3. Results and discussion

3.1. $CaCO_3$ buffered ABE fermentation (uncontrolled pH)

Three different initial glucose concentrations (33, 66 and 100 g L^{-1}) were used as the substrate for the ABE fermentation under 5 g L^{-1} $CaCO_3$ as a buffering agent. These initial sugar concentrations were chosen within the typical range of sugar production after pre-treatment and enzymatic hydrolysis of lignocellulosic biomass. Sugar concentrations below ~45 g L^{-1} are usually obtained, although concentrations techniques may be applied to increase them [3]. The fermentation profiles are shown in Fig. 2. The $CaCO_3$ was able to buffer the fermentation media and keep the minimum pH around 4.6 in all the experiments. The maximum production of acetic and butyric acids was between 5 and 6 g L^{-1} in all the tests. In fact, high acid concentration can be related to the presence of $CaCO_3$. Ren et al. [32] obtained ~6-fold more acetic and ~1.4-fold more butyric when culturing *C. acetobutylicum* ATCC 824 in P2 media supplemented with $CaCO_3$ than supplementing with P2 media only. $CaCO_3$ could stimulate the acid production by the stabilization of the cell membrane proteins due to the presence of divalent ions (Mg^{+2} and Ca^{+2}) [33]. The three experiments had almost the same biomass production. The glucose consumption rate and maximum growth rate were nearly the same as the average values of 2.09 ± 0.15 g L^{-1} h^{-1} and 0.197 ± 0.008 h^{-1} respectively. However, the test with the lowest initial glucose experiment (33 g L^{-1}) showed some differences in the ABE fermentation profile and product yields.

In the experiment with 33 g L^{-1} of initial glucose, nearly all the glucose was depleted in the first 24 h, accompanied by increased butanol production (~2.64 g L^{-1} at 28 h). However, the rapid glucose depletion limited, to some extent, the assimilation of butyric acid to butanol, giving a final butanol concentration of 3.35 g L^{-1} (ABE concentration: 3.99 g L^{-1}). Butyric acid remained at a high concentration (~6.0–6.6 g L^{-1}) from 15 h, and glucose depletion kept the butyric acid assimilation rate lower than its production rate. At the glucose level of 33 g L^{-1}, maximum butanol and ABE yields of 0.099 and 0.118 g g^{-1},

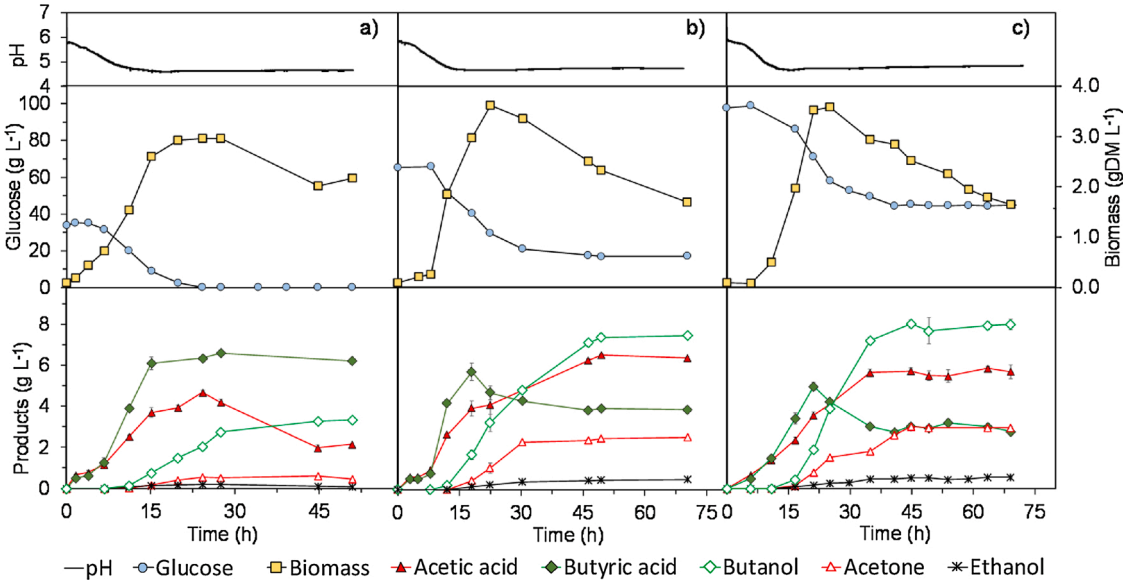


Fig. 2. Effect of initial glucose concentration on butanol production under uncontrolled pH by *C. acetobutylicum* (a, 33; b, 66; c, 100 g L^{-1} of initial glucose).

respectively, were obtained (Table 1). Similar solvent yields have been reported in previous studies. For example, Ibrahim et al. [12] obtained a butanol (ABE) yield of 0.11 (0.15) and 0.13 (0.22) with 20 and 40 g L^{-1} of glucose with *C. acetobutylicum* ATCC 824 without pH control. In Long et al. [34], who tested gradually increasing glucose concentrations, the *C. acetobutylicum* P262 strain produced ~1, ~4, ~12 and ~13 g L^{-1} of ABE with 20, 30, 40 and 60 g L^{-1} of glucose, respectively, indicating that >40 g L^{-1} of glucose is required to achieve ABE yields >0.20. However, other *Clostridium* species may behave differently under the same glucose concentrations. For example, *C. beijerinckii* P260 produced 8.0 (11.5) g L^{-1} of butanol (ABE) with 29.3 g L^{-1} of initial glucose concentration without pH control [8]. Our results are thus important for adequate planning the conversion of butanol from lignocellulosic waste using *C. acetobutylicum*.

However, the reactors with 66 and 100 g L^{-1} achieved butanol (ABE) concentrations of 7.47 (10.50) and 8.01 (11.59) g L^{-1} respectively (Table 1). These titers gave a ~2.3 (~2.8)-fold increase in butanol (ABE) production when compared with 33 g L^{-1} of initial glucose concentration. In addition, the butanol (ABE) yields were 0.155 (0.217) and 0.151 (0.220) g g^{-1} for 66 and 100 g L^{-1} respectively (Table 1), an increase of ~54 % (~85 %) versus the 33 g L^{-1} of the initial glucose experiment. A minimum pH of ~4.65 was achieved at ~16–18 h, with an acid production (acetic plus butyric) of 9.63 (at 18 h) for 66 g L^{-1} of initial glucose and 8.54 g L^{-1} (at 21 h) for the 100 g L^{-1} reactor. Also, ~2 g L^{-1} of butyric acid was reassimilated in both reactors from the acid peak concentration until the end of the fermentation. The better performance of these two reactors than the lowest initial glucose experiment is associated with the residual glucose remaining when the minimum pH was reached (near the end of the acidogenic stage). A glucose concentration higher than 40 g L^{-1} (at ~16–18 h) was sufficient to develop the solventogenic stage in both reactors. However, the glucose was not completely depleted in either reactor, with only 74 % of the glucose consumed in the 66 g L^{-1} experiment and 54 % in the 100 g L^{-1}. Glucose consumption sharply declined after 12 h from the point at which the butyric acid peaked (Fig. 2). This could be associated with the high undissociated acid concentrations reached when the acids peaked (63 and 53 mM for 66 and 100 g L^{-1} of initial glucose reactors, respectively). Accordingly to Maddox et al. [26], acid crash phenomenon can occur when the upper limit of total undissociated acid concentration (57–60 mM) is exceeded. Other authors have obtained better butanol production for this strain in similar environmental conditions, but with lower initial glucose concentrations. For example, Yang et al. [23] reported a butanol production of 10.8 g L^{-1} (butanol yield: 0.216 g g^{-1}) with 50 g L^{-1} of initial glucose with the same minimum pH (4.7) using *C. acetobutylicum* ATCC 824 and 5 g L^{-1} of $CaCO_3$ as a buffer. The lower butanol production and yield obtained in our experiment seems to indicate that the spontaneous ABE fermentation without pH control could facilitate conditions for acid crash when the initial glucose concentrations is higher than 60 g L^{-1}. The undissociated form of the acid is more toxic than the dissociated, as the former species can diffuse through the plasma membrane to the cytosol, where it dissociates and reduces the intracellular pH [35]. An active pH control could thus help to limit the concentration of undissociated acid species as a strategy to improve butanol production.

3.2. Fermentations under active pH control

3.2.1. Screening of the set-point for minimum pH

In order to avoid acid crash, a pre-screening experiment was carried out to evaluate the fermentation profiles under different minimum controlled pHs and using 60 g L^{-1} of initial glucose. The minimum pH tested were 4.8; 5.0; 5.1 and 5.5. The chosen pH_{min} control strategy was selected to optimize both butanol production and glucose consumption. The results indicate that controlling the pH_{min} led to reduced total undissociated acid concentration in the reactors. The maximum concentrations (total acetic and butyric acids) were 50.61, 42.03, 37.64 and 39.46 mM for the reactors with the minimum pH of 4.8, 5.0, 5.1 and 5.5 respectively. Controlling the pH thus kept the undissociated acid values below the levels related to acid crash. In addition, complete glucose depletion occurred in all the conditions tested, even though increasing the pH_{min} from 4.8 to 5.5 led to a higher glucose consumption rate, from 1.90 to 3.47 g L^{-1} h^{-1} (Table 2). The maximum growth rates were close within any of the pH studied, with an average value of 0.202 ± 0.006 h^{-1}, similar to the reactors with no active pH control (Table 1). When pH_{min} was 5.5, the main fermentation product was butyric acid (15.88 g L^{-1}), indicating acidogenic fermentation. In contrast, solventogenesis failed to occur and butanol production was almost residual (2.01 g L^{-1}, yield: 0.031 g g^{-1}) (Table 2). In fact, the acidogenic fermentation could have been related to the residual glucose concentration of 6 g L^{-1} at 20 h, which may be below the limit for developing solventogenesis. The reactors at 5.1, 5.0 and 4.8 pH reported similar butanol (ABE) productions of 11.22 (16.12), 11.42 (16.67) and 11.57 (18.27) g L^{-1} respectively. ABE yield was slightly higher at pH of 4.8 (0.301 g g^{-1}) as more ABE was produced. A ~1.5-fold increase in butanol production was obtained over the same initial glucose concentration without pH control. Little data is available in the literature on comparing the effect of different values of pH on *Clostridium* species. Jiang et al. [36] evaluated a set of pH values from 4.9 to 6.0 for *C. beijerinckii* IB4 with 60 g L^{-1} of initial glucose. This strain showed an optimum pH of 5.5 for butanol production and glucose consumption rate. Other pH may be set for ABE fermentation of other *Clostridium* species. For example, a value of 4.8 was successfully stablished for *C. beijerinckii* DSM 6423 [37] and a value of 5.5 for *C. saccharoperbutylacetonicum* N1–4 [38]. Our results showed that when pH_{min} was controlled, solvent production and glucose consumption rate outperformed the uncontrolled pH experiment. A pH_{min} of 5.1 was therefore selected for the next experiments as this value showed the highest glucose consumption rate (~3.0 g L^{-1} h^{-1}) among similar butanol titers (~11 g L^{-1}).

Table 2
Influence of the minimum controlled pH on production and kinetics parameters. Initial concentration of glucose: 60 g L^{-1}. Yields were calculated with the maximum solvent concentration.

pH	$Butanol_{max}$ (g L^{-1})	ABE_{max} (g L^{-1})	$Y_{B/S}$ (g g^{-1})	$Y_{ABE/S}$ (g g^{-1})	μ_{max} (h^{-1})	$-q_{glucose}$ (g L^{-1} h^{-1})
5.5	2.01	2.92	0.031	0.046	0.204	3.47
5.1	11.22	16.12	0.163	0.235	0.205	3.02
5.0	11.42	16.67	0.179	0.256	0.194	2.61
4.8	11.57	18.27	0.186	0.301	0.206	1.90

Table 1
Production and kinetics parameters under uncontrolled pH (natural evolution of pH). Yields were calculated with the maximum solvent concentration. Glucose conversion refers to the end of fermentation.

$C_{glucose}$ (g L^{-1})	$Butanol_{max}$ (g L^{-1})	ABE_{max} (g L^{-1})	$Y_{B/S}$ (g g^{-1})	$Y_{ABE/S}$ (g g^{-1})	μ_{max} (h^{-1})	$-q_{glucose}$ (g L^{-1} h^{-1})	Glucose conversion (%)
33	3.35	3.99	0.099	0.118	0.188	2.27	100
66	7.47	10.50	0.155	0.217	0.202	2.00	74
100	8.01	11.59	0.151	0.220	0.202	2.02	54

3.2.2. Combined effect of initial glucose concentration and the minimum pH control

Batch fermentations keeping pH_{min} at 5.1 were performed with glucose concentrations of 33, 66 and 100 g L^{-1} to evaluate the combined effect of controlled pH and the initial glucose concentration on the fermentation process. The profiles of these fermentations are shown in Fig. 3 and the main representative parameters are given in Table 3. The use of a pH control clearly modified the pH profiles as compared to their respective uncontrolled pH fermentations (Fig. 2). At 5.1, the maximum undissociated acid concentration obtained in the 33, 66 and 100 g L^{-1} of the initial glucose reactors were 43.88; 37.64 and 36.15 mM, respectively, a reduction of about 30–40 % in comparison with the non-controlled pH experiments. None of the controlled reactors therefore reached the value of 57−60 mM of total undissociated acids in the media that could cause an acid crash. The maximum biomass concentration in the two reactors with the highest initial glucose concentration (66 and 100 g L^{-1}) were ~1.5-fold higher than the reactor with 33 g L^{-1} of initial glucose. As expected from the pre-screening experiment, active pH_{min} control resulted in higher glucose consumption rate values (average: 3.25 ± 0.21 g L^{-1} h^{-1}) than the uncontrolled reactors (average: 2.09 ± 0.15 g L^{-1} h^{-1}). A similar growth rate was observed in previous experiments with a maximum average growth rate of 0.200 ± 0.009 h^{-1} (Table 3). No significant differences were obtained between the three sets of experiments (uncontrolled, pre-screening and pH_{min} at 5.1) (ANOVA p-value: 0.82), indicating that the maximum growth rate value may be used as a checkpoint to determine whether the culture has developed correctly during a sequential series of experiments. In any case, as maximum growth rates are culture dependent they must be determined *in situ*. For example, Liao et al. [39] and Buendia-Kandia et al. [40] found values of ~0.18 and ~0.26 h^{-1} respectively, when *C. acetobutylicum* ATCC 824 was grown in different media formulations.

The ABE fermentation profile of 33 g L^{-1} initial glucose showed a failure in solvent production (Fig. 3). A redistribution of the carbon flux was observed as the main fermentation product was butyric acid (9.43 g L^{-1}) and the glucose was exhausted at ~17 h. In fact, maximum butyric acid concentration increased by ~1.5-fold over the uncontrolled reactor. Butanol (ABE) production was 1.09 (1.59) g L^{-1} and butanol (ABE) yield was 0.032 (0.047) g g^{-1} (Table 3). These solvent productions were on average ~0.4-fold less butanol, acetone and ABE concentration than the uncontrolled pH assay, although ethanol production increased from 0.18 to 0.26 g L^{-1}. The yields with 33 g L^{-1} of initial glucose were almost identical to those obtained in the controlled fermentation at pH 5.5, in which the glucose was completely exhausted (Screening pH Section, Table 2). The low residual glucose in the early stages of fermentation could explain the failure to switch to solventogenesis and the acidogenic fermentation. *C. acetobutylicum* DSM 792 may not be the most suitable when working at low sugars concentrations, although, Dolejš et al. [41] were able to produce 2.7 g L^{-1} of butanol with 5.0 pH and 20 g L^{-1} initial glucose concentration using *C. acetobutylicum* DSM 1731.

A minimum glucose concentration may be necessary to properly develop ABE fermentation with pH_{min} at 5.1. Butanol production in the reactors with 66 and 100 g L^{-1} initial glucose was 11.22 and 13.83 g L^{-1}, respectively. These values were a ~1.5 and ~1.7-fold increase over their counterparts with no pH control. Indeed, acetone and ethanol increased an average of ~1.6-fold and ~1.8-fold, respectively, when pH control was applied. This indicates the feasibility of limiting the pH_{min} to 5.1 to improve solvent titers when sufficient carbon source is available for *C. acetobutylicum* DSM 792. When a similar strategy was recently applied by Luo et al. [31], they obtained 9.42 g L^{-1} of butanol with pH set at 5.0 during acidogenesis using 60 g L^{-1} of initial glucose with *C. acetobutylicum* ATCC 824. Our results confirm that a suitable pH control strategy can increase the butanol titer in ABE fermentation. Butanol and ABE yields were almost identical, with 66 and 100 g L^{-1} of initial glucose concentration (Table 3), although more butanol was produced when using 100 g L^{-1} of glucose. In fact, maximum butyric acid was 6.89 g L^{-1} (at ~18 h) for the 66 g L^{-1} reactor and 7.11 g L^{-1} (at ~21 h) for the 100 g L^{-1}, these concentrations represent an average ~1.3-fold increase over uncontrolled pH control fermentations. Furthermore, pH was recovered in both reactors as butyric acid was assimilated. A similar butyric acid concentration of ~2.60−2.80 g L^{-1} remained in both reactors at the fermentation end point, indicating a ~2.1-fold increase in butyric acid reassimilation with pH control over the uncontrolled pH experiment. Complete glucose depletion was achieved in the fermentation with 66 g L^{-1} of initial glucose, while the fermentation with 100 g L^{-1} of initial glucose consumed 84.9 g L^{-1} (87 % of total glucose). Therefore, an increase of 42 % and 59 % in glucose consumption with 66 and 100 g L^{-1} of initial glucose, respectively, was achieved versus the no pH control assays. High initial glucose concentrations may induce substrate inhibition in the *Clostridium* species. For example, Qureshi and Blaschek [42] found substrate inhibition for *C. beijerinckii* with 158 g L^{-1} of initial glucose. In our study, no substrate inhibition was found at glucose concentrations as high as 100 g L^{-1}, as similar biomass was obtained in reactors with 66 and 100 g L^{-1} of initial glucose concentration. Due to the higher butanol production in the study

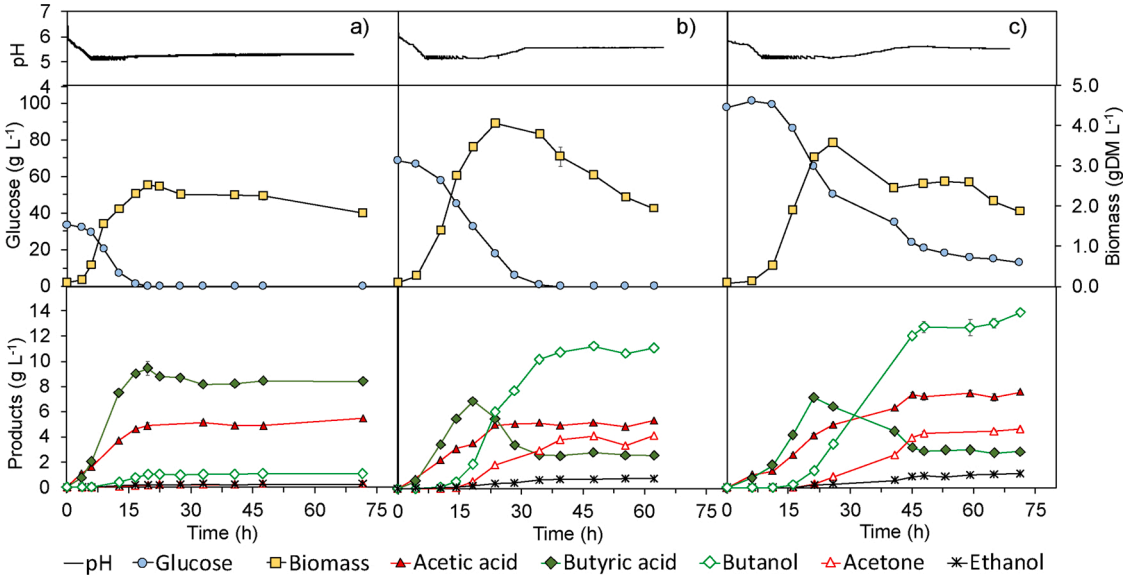


Fig. 3. Effect of initial glucose concentration on butanol production with pH_{min} at 5.1 by *C. acetobutylicum* (a, 33; b, 66; c, 100 g L^{-1} of initial glucose).

Table 3
Production and kinetics parameters under controlled pH (at $pH_{min} \geq 5.1$). Yields were calculated with the maximum solvent concentration. Glucose conversion refers to the end of fermentation.

$C_{glucose}$ ($g\,L^{-1}$)	$Butanol_{max}$ ($g\,L^{-1}$)	ABE_{max} ($g\,L^{-1}$)	$Y_{B/S}$ ($g\,g^{-1}$)	$Y_{ABE/S}$ ($g\,g^{-1}$)	μ_{max} (h^{-1})	$-q_{glucose}$ ($g\,L^{-1}\,h^{-1}$)	Glucose conversion (%)
33	1.09	1.59	0.032	0.047	0.205	3.35	100
66	11.22	16.12	0.163	0.235	0.205	3.02	100
100	13.83	19.55	0.163	0.232	0.190	3.34	87

with 100 g L^{-1} of initial glucose, a concentration step for sugars in lignocellulosic hydrolysates could be a suitable option to enhance the economic viability of ABE fermentation.

3.3. Hybrid pH strategy

A hybrid pH control strategy was proposed and tested for the three initial glucose concentrations (33, 66 and 100 g L^{-1}). This strategy consisted of spontaneous pH evolution until reaching minimum pH, followed by a sudden pH step control up to 5.1 at ~21–24 h, and from then on keeping pH_{min} at 5.1. In our reactors with 33 and 66 g L^{-1} of initial glucose, butanol production with the hybrid pH strategy was lower than either uncontrolled pH (2.56 g L^{-1}) or pH_{min} at 5.1 (9.08 g L^{-1}). On the other hand, when higher initial sugars concentrations were used, better fermentation results were found by implementing this pH strategy. This type of pH modification strategy may rely on culture conditions such as growth phase or carbon source availability at the modification time. The results of the fermentation of 100 g L^{-1} of initial glucose under the hybrid pH control strategy are shown in Fig. 4. The butanol and ABE production for the assay with 100 g L^{-1} of initial glucose was 15.80 and 23.68 g L^{-1} respectively. An increase of ~1.1 and ~1.2-fold in butanol and ABE production over to the experiment carried out at 5.1 pH_{min} with the same initial glucose. Although butanol inhibition could occur when more than 14 g L^{-1} were present in the media when using *C. acetobutylicum* ATCC 824 [43], our data indicate that a delay in cell death may allow this limit to be exceeded.

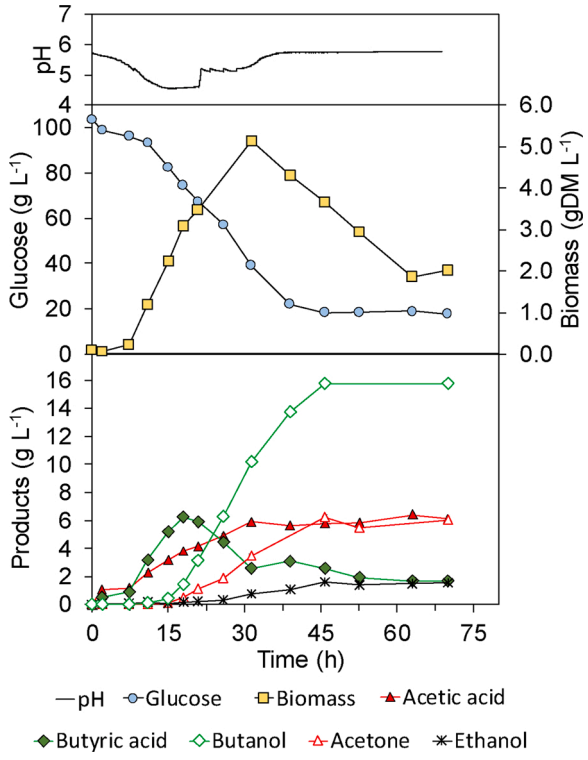


Fig. 4. Batch fermentation on butanol production under hybrid pH control with 100 g L^{-1} of initial glucose concentration by *C. acetobutylicum*.

The maximum butyric acid of 6.25 g L^{-1} was obtained at ~18 h with a pH of 4.57. At this time-point the total undissociated acids concentration was 71.73 mM, which could have caused an acid crash. Even though the pH was increased to 5.1 at ~21 h, the fermentation was able to avoid the acid crash. Indeed, butyric acid was reassimilated during solventogenesis with a final concentration as low as 1.68 g L^{-1} (~1.2-fold increase over the reactor with pH_{min} at 5.1). In this assay 85.9 g L^{-1} of glucose (83 % of total) was consumed, thus showing no improvement in carbon source uptake over the experiment with pH_{min} at 5.1. The glucose consumption rate was 2.55 g L^{-1} h^{-1}, an intermediate value between the no pH control and the pH_{min} control at 5.1. The maximum growth rate was 0.223 h^{-1} which matched the values previously obtained. The highest biomass concentration was achieved in these conditions, reaching a concentration of 3.95 g L^{-1} at ~31 h. A delay of 6–8 h in the maximum concentration was found, which was attributed to the activation of the pH control at 21 h (time point when biomass nearly peaked in previous experiments, Fig. 3). The time-point recommended for the sudden pH step may thus be at the mid-final exponential growth phase when the minimum pH is reached. These results show that fermentation pH has a direct impact on butanol biosynthesis and glucose uptake, however, this pH strategy might be difficult to carry out as it is dependent on time and acid concentration. Indeed, glucose availability could be a key factor in developing a proper pH control strategy. For example, Yang et al. [23] carried out a time dependent (12, 24 and 36 h) pH control procedure for 50 g L^{-1} of glucose and butanol production was only improved (~2.1-fold higher) when the pH was modified at 12 h. Our results indicate that for glucose levels of ~50–70 g L^{-1}, using pH_{min} is a more robust strategy than implementing a time dependent pH control set-points.

This work has shown that the best pH control strategy depends on the initial glucose level (low, medium and high). At the low level (33 g L^{-1} of initial glucose) uncontrolled pH was more efficient, while at the medium level (66 g L^{-1} of initial glucose) using controlled pH_{min} was the best alternative. Lastly, at the high level (100 g L^{-1} of initial glucose) the hybrid pH strategy provided the highest butanol production. These promising results might be validated under more realistic conditions by checking the effect of other sugars or potential inhibitors on product yields. Future research will therefore focus on testing these pH control strategies with lignocellulosic wastes hydrolysates.

4. Conclusion

This study aimed to determine the most favourable pH control strategy for maximizing butanol production and glucose consumption by *C. acetobutylicum* DSM 792 within a broad range of initial glucose concentrations. For an initial glucose concentration of 33 g L^{-1}, formulating the media with an alkalinity buffer was shown to be the best alternative to enhance butanol production. For the experiments with 33 g L^{-1}, lower butanol concentration was achieved, indicating that this strain needs a higher sugar concentration to compete with other strains such as *C. beijerinckii*. Otherwise, when enough glucose is available (>66 g L^{-1}), the results indicate the superiority of controlling minimum pH as a way of increasing solvent production in ABE fermentation. When high sugar concentrations are present (100 g L^{-1}) a more sophisticated pH control strategy coupled with the acid production pattern can

substantially improve the butanol titer. These results are of practical interest for establishing *ad-hoc* strategies in ABE fermentation from lignocellulosic waste, as the best pH control strategy has been shown to depend on initial sugar concentration. The experimental process proposed here can also be extended to other *Clostridium* species.

CRediT authorship contribution statement

M. Capilla: Investigation, Formal analysis, Writing - original draft, Visualization. **P. San-Valero:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision. **M. Izquierdo:** Conceptualization, Methodology, Visualization. **J.M. Penya-roja:** Conceptualization, Methodology, Resources. **C. Gabaldón:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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The Influence of Sugar Composition and pH Regulation in Batch and Continuous Acetone–Butanol–Ethanol Fermentation

Miguel Capilla, Carlos Silvestre, Alejo Valles, Francisco Javier Álvarez-Hornos, Pau San-Valero * and Carmen Gabaldón

Research Group in Environmental Engineering (GI2AM), Department of Chemical Engineering, University of Valencia, 46100 Burjassot, Spain; miguel.capilla@uv.es (M.C.); carlos.silvestre@uv.es (C.S.); alejo.valles@uv.es (A.V.); francisco.j.alvarez@uv.es (F.J.Á.-H.); carmen.gabaldon@uv.es (C.G.)
* Correspondence: pau.valero@uv.es; Tel.: +34-963-543-451

Abstract: Acetone–butanol–ethanol (ABE) fermentation is influenced by external conditions. This work aimed to study the influence of pH regulation on monosaccharide composition in batch and continuous fermentation processes to determine butanol production and productivity. Batch fermentations with ammonium acetate or calcium carbonate combined with minimum pH control (pH $\geq$ 4.8 or 5.1) were assessed with pure xylose and glucose/xylose mixtures (ratios of 1:1 and 3:1). Continuous two-stage fermentation was developed using plastic rings to retain the biomass. Although batch fermentations with pure xylose performed better without active minimum pH control with both buffers, minimum pH control was necessary to metabolize xylose in the presence of glucose. Xylose uptake was favored by the use of calcium carbonate and pH $\geq$ 5.1 at a ratio of 1:1, while ammonium acetate and a pH $\geq$ 4.8 was the best option for a 3:1 ratio. The best butanol production and productivity values with sugar mixtures in batch reactors were 8.8 g L^{-1} and 0.61 g L^{-1} h^{-1} with an ammonium acetate pH $\geq$ 4.8 (ratio 3:1). The glucose/xylose ratio combined with pH regulation thus modulated xylose metabolism and solvent production in batch modes. Immobilized cells combined with operating at D = 0.333 h^{-1} and pH regulation increased butanol productivity almost fourfold up to 2.4 $\pm$ 0.2 g L^{-1} h^{-1}.

Keywords: ABE fermentation; pH control; calcium carbonate; ammonium acetate; monosaccharides; continuous fermentation

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1. Introduction

Interest in the development of biofuels has greatly increased due to the rapid depletion of fossil fuels and the rising production of greenhouse gases [1]. Because of its better physicochemical properties than other biofuels such as ethanol, biobutanol is being widely investigated [2]. Biobutanol can be transported and delivered by the existing infrastructures, which would reduce the overall cost of implementation [1,3]. It can be produced by solventogenic *Clostridium* via acetone–butanol–ethanol (ABE) fermentation, which can use monosaccharides from lignocellulosic biomasses, mainly glucose and xylose, to obtain second generation biobutanol [4].

However, the microorganism is not efficiently capable of uptaking different sugars simultaneously due to carbon catabolite repression (CCR) [5]. This phenomenon can be a problem, as it may cause sequential sugar uptake and increase residence time and overall costs. To overcome this drawback, several measures have been considered, such as fermentation conditions or metabolic engineering [6]. The operational conditions may change the outcome of the ABE fermentation; parameters such as carbon source (cellobiose, lactose or glucose) [7–9], media formulation (buffer components, nitrogen source or metals) [10–12] or pH [13] play an important role in developing and producing bacteria.

The combination of acid overproduction and low pH can lead to acid crash due to an undissociated acid concentration boost. These undissociated acid species can permeate

through the cell, preventing sugar uptake and solvent production [14]. To avoid this, pH can be controlled by buffering compounds such as calcium carbonate [15] or ammonium acetate [16]. Focused chemical control strategies can also be used to regulate the pH value with *C. acetobutylicum*. Batch fermentations are the easiest and most conventional configurations for ABE fermentation [1]. For example, batch modes with different levels of initial glucose (33, 66 and 100 g L^{-1}) [17] and with a two-stage pH control strategy [18] or with lignin-derived inhibitors from lignocellulosic pretreatment [19] have been tested.

In contrast, although continuous fermentation requires close process control, it allows increased productivity and sophisticated capabilities in the fermentation process [20]. The recent implementation of cell immobilization, which is under investigation mainly using glucose systems [21,22], has certain advantages, such as prevention of microorganism washout, uncoupling the dilution rate and the maximum growth rate or minimizing the propagation cost. However, there are other disadvantages such as clogging or mass-transfer limitations [20].

In the present work, a study was made of the influence of pH regulation on monosaccharide composition to evaluate butanol production and productivity in ABE fermentation. To assess the shared effect of buffer formulation and active pH control with pure xylose and glucose/xylose ratios (1:1 and 3:1), a novel pH-controlled continuous cell retention ABE fermentation was carried out in the optimal conditions of batch fermentations to further increase butanol productivity.

2. Materials and Methods

2.1. Microorganism and Medium Fermentation

C. acetobutylicum DSM 792 was acquired from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and maintained in 20% glycerol at −80 °C. For seed inoculum, the bacteria were statically cultured at 37° in 19 g L^{-1} of Reinforced Clostridial Medium (RCM) supplemented with 10 g L^{-1} of glucose. The sterilized fermentation medium contained (g L^{-1}): sugars, 60 (glucose, xylose or sugar mixture); yeast extract, 5; KH_2PO_4, 0.5; K_2HPO_4, 0.5; resazurin sodium salt, 0.001; antifoam 204, 0.01%; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 7H_2O$, 0.01; and $FeSO_4 \cdot 7H_2O$, 0.05. Two buffers were studied in batch fermentations: ammonium acetate and calcium carbonate bases. The buffers tested were ammonium acetate, 2.2 g L^{-1} or calcium carbonate, 5 g L^{-1} combined with NH_4Cl, 2 g L^{-1}. The chemicals were purchased from VWR, except for $CaCO_3$ (Merk, Darmstadt, Germany), antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA) and yeast extract (Alfa Aesar, Haverhill, MA, USA).

2.2. Experimental Setup

2.2.1. Batch Fermentations

Batch fermentations were carried out using 0.8 L of effective volume in a 1.1 L total volume reactor (Figure 1A). Anaerobic conditions were accomplished before inoculation by insufflating N_2 for 30 min. The assays were carried out with an inoculum size of 5% at 120 rpm and 37 °C. The fermentations were monitored by a Tris-compatible flat pH sensor connected to LoggerPro software (Vernier, Beaverton, OR, USA). A feedback control strategy on/off was used to avoid the pH decreasing below a set point value (4.8 or 5.1 depending on the experiment). NaOH (3 M) was automatically added when the pH reached the lower limit (dead band 0.1) (Figure 1C). The upper limit was not controlled, allowing the recovery of the pH when possible. Samples were collected at specific time-points to analyze cell growth and metabolites.

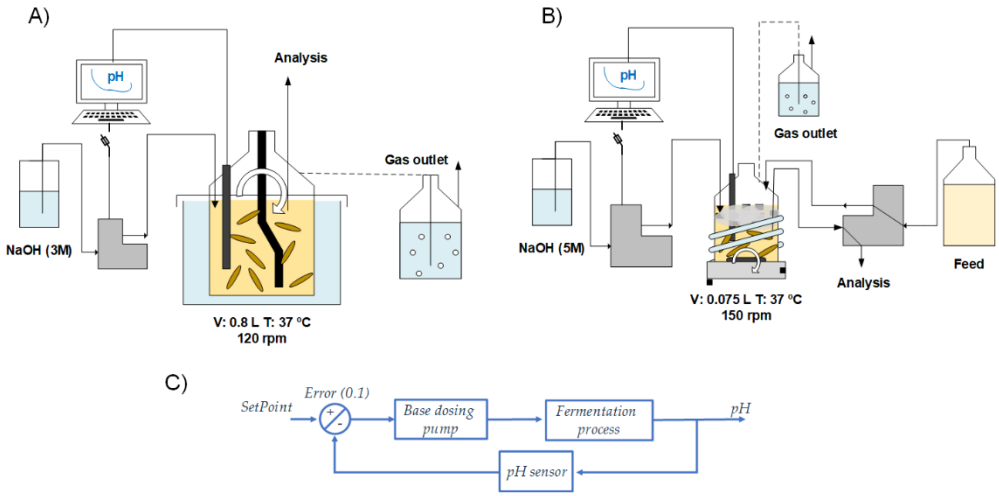

Figure 1. Fermentations setup with *C. acetobutylicum* DSM 792. (**A**) Batch fermentations, (**B**) continuous fermentation and (**C**) block diagram for the pH.

The experimental plan is summarized in Table 1. The use of xylose was tested as it is the second main monosaccharide in lignocellulosic hydrolysates. The reduction of xylose concentration concomitant with incrementing the glucose was used to evaluate the effect of monosaccharide composition which would mimic a spectrum of lignocellulosic hydrolysates concentrations. In that sense, three different glucose/xylose mass ratios (0:1, 1:1, 3:1, final monosaccharide concentration: 60 g L^{-1}) were tested in batch mode. For every sugar composition, the effect of buffer coupled or not with a strategy of limiting the minimum pH was evaluated. Both buffers were selected as they previously demonstrated good performance in ABE fermentation [10,17]. The set points were chosen from a previous work with glucose [17].

Table 1. Experimental plan with batch mode. The value of pH control indicates the minimum pH reached.

Glucose/Xylose Ratio	Ammonium Acetate (2.2 g L^{-1})		Calcium Carbonate (5 g L^{-1})	
	Blank	pH Control	Blank	pH Control
0:1	Yes	4.8	Yes	5.1
1:1	Yes	4.8	Yes	5.1
3:1	Yes	4.8	Yes	4.8 and 5.1

2.2.2. Continuous Fermentation

A stirred continuous fermentation was carried out using 0.075 L of effective medium in a 0.25 L reactor (Figure 1B). The medium was prepared as described previously with ammonium acetate as buffer. The configuration used a multichannel peristaltic pump (Reglo ICC, Ismatec, Wertheim, Germany) at the desired equal in-and-out flow rate. Plastic rings were used as packing material at 7.5% *w*/*v*. Before inoculation, anaerobic conditions were accomplished by insufflating N_2 for 10 min. The fermentation was carried out with an inoculum size of 5% at 150 rpm and 37 °C. The temperature was maintained by a water heating coil while stirring with a magnetic stirrer. An identical pH monitoring protocol was used as in the batch fermentations based on the automatic limitation of minimum pH. A two-stage pH reactor was developed by chemically controlling the fermentation at two different minimum set points (6.0 and 4.8) using NaOH (5 M). The summary conditions of the experiment are shown in Table 2. Glucose was used as carbon source and pH $\geq$ 6.0 to favor acidogenesis conditions in the biomass growth phase, performing the batch mode in the first 30 h, after which the continuous mode started (dilution rate, D = 0.042 h^{-1}). During

the acidogenesis phase, the dilution rate was doubled every ~48 h up to at D = 0.166 h^{-1} to improve productivity. After that, at 144 h, the pH was allowed to drop to pH $\geq$ 4.8 to favor the solventogenesis phase.

Table 2. Summary conditions of the continuous fermentation.

Time (h)	Minimum pH Set Point	Substrate	Concentration (g L^{-1})	D (h^{-1})
0–30	6.0	Glucose	60	Batch
30–72	6.0	Glucose	60	0.042
72–121	6.0	Glucose	60	0.083
121–144	6.0	Glucose	60	0.167
144–192	4.8	Glucose	60	0.167
192–216	4.8	Glucose	60	0.333
216–288	4.8	Glucose/Xylose	40:20	0.333

2.3. Analytical Methods

Suspension cell density (gDW L^{-1}) was quantified at 600 nm (OD_{600}) using a UV–Vis spectrophotometer (SpectroFlex 6600, WTW, Weilheim in Oberbayern, Germany) as gDW L^{-1} = 0.2941·OD_{600} + 0.0331 (R^2 = 0.9908). Samples were centrifuged (10,000 rpm, 5 min) and 0.22 µm filtered. Metabolites of interest were quantified using liquid chromatography (Agilent 1100 Series HPLC system, Agilent Technologies, Santa Clara, CA, USA) coupled with an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 50 °C. A refractive index detector (RID) at 35 °C was utilized to detect monosaccharides, ethanol and butanol, while a diode array detector (DAD) was used at 210 nm to detect butyric and acetic acids and at 280 nm to detect acetone. Five mM of sulfuric acid was used as mobile phase at 0.6 mL min^{-1} for 40 min. The Henderson–Hasselbalch formula was used to quantify the undissociated acids (pK_aacetic: 4.76; pK_abutyric: 4.82). The maximum production rates in batch were estimated at the exponential phase of each compound by Equation (1):

$$r = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \quad (1)$$

where r is the production rate (g L^{-1} h^{-1}). S_{t1} and S_{t2} are the species concentrations at the initial and final point of the exponential phase (g L^{-1}), and t_2 and t_1 are the starting and ending times of the exponential phase (h). For continuous fermentations, productivity was estimated as follows:

$$P_s = S \cdot D \quad (2)$$

where S is the average species production (g L^{-1}), and D is the dilution rate (h^{-1}).

3. Results and Discussion

3.1. Batch Fermentation

Two buffer media (calcium carbonate/ammonium acetate) and their combination with an active pH control were compared for 60 g L^{-1} of xylose (Figure 2). The evolution of the pH is shown in Figure S1A. The growth of the bacteria using xylose was characterized by an initial lag phase of 12–24 h, after which the exponential growth started (Figure 2G). The use of calcium carbonate increased biomass concentration 1.4-fold over the ammonium acetate in spontaneous fermentation, indicating that calcium carbonate enhanced biomass production. The combination of buffer and pH control clearly changed the biomass pattern; the maximum biomasses increased from 4.2 to 7.2 g L^{-1} for the acetate reactors (blank and pH $\geq$ 4.8) and from 5.8 to 7.9 g L^{-1} for the carbonate reactor (blank and pH $\geq$ 5.1). This increase represented about ~1.7 and 1.4 times with ammonium acetate and calcium carbonate, respectively. Similar xylose consumptions were obtained whatever the buffer used and the pH set point employed, with no complete depletion of the monosaccharide

(Figure 2A). This was because a butanol concentration of ~4.50 g L^{-1} would reduce the initial xylose uptake rate by 50%. A titer of ~8.0–8.5 g L^{-1} has been determined to be growth inhibitory for *C. acetobutylicum* on xylose as the carbon source [11,23]. The impact of the buffer can be seen in the butyric acid concentration (Figure 2E). Calcium carbonate almost doubled (6.6 over 3.4 g L^{-1}) the maximum concentration of the acid against ammonium acetate with no pH control. Under the conditions tested with xylose as the sole carbon source, both buffers performed better with spontaneous fermentation without pH control. Butanol (ABE) productions were 8.0 (13.1) and 5.3 (8.1) g L^{-1} for the acetate reactor (blank and pH $\geq$ 4.8) while 7.8 (11.8) and 5.8 (8.1) g L^{-1} for the carbonate reactor (blank and pH $\geq$ 5.1). These results show that the minimum pH control was detrimental with either ammonium acetate or calcium carbonate buffers under these conditions. Previous results showed that when 60 g L^{-1} glucose was used as a sole carbon source, the strategy of limiting the minimum pH at 5.1 enhanced butanol (ABE) production from 7.47 (10.50) g L^{-1} to 11.22 (16.12) g L−1 [17]. As xylose uptake behaved differently to glucose, which is the major monosaccharide of lignocellulosic residues, the effect pH regulation (active pH control and/or buffer media) with its mixtures should be investigated.

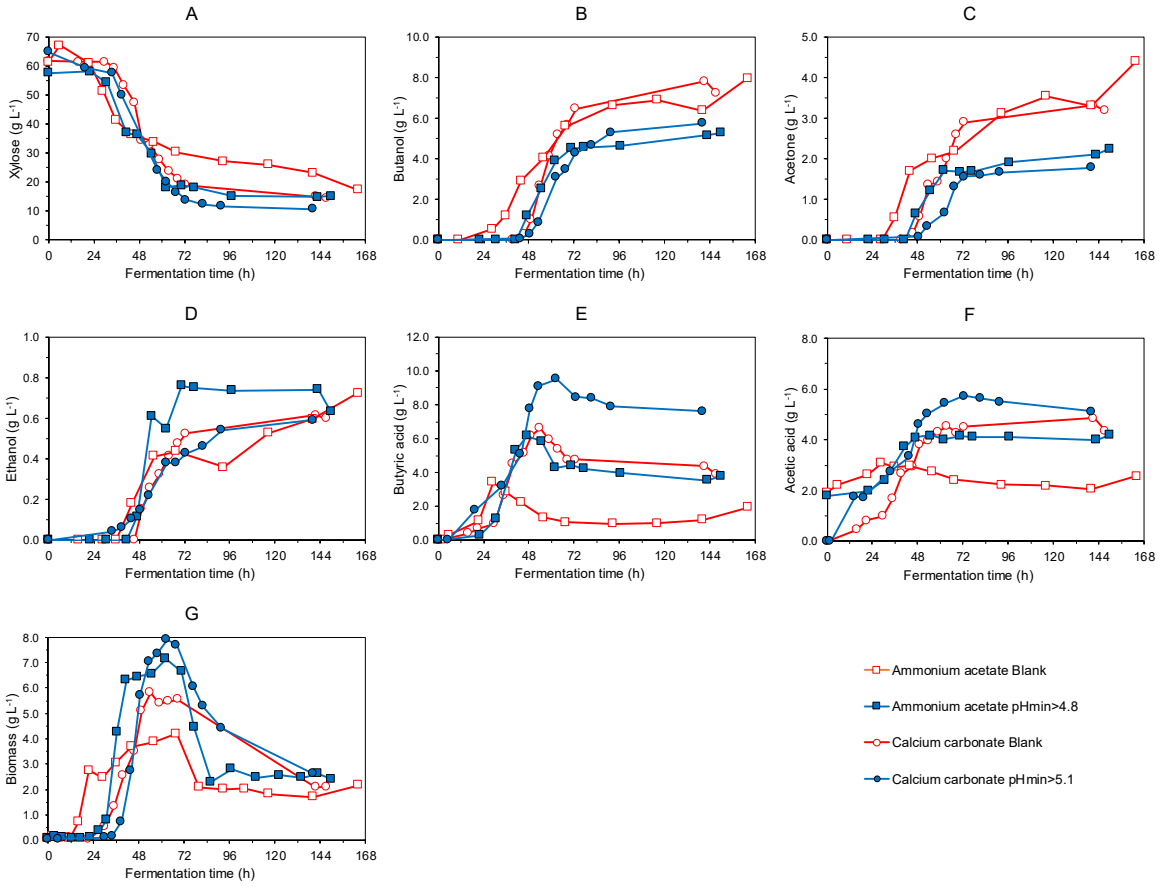

Figure 2. Fermentation profiles of (**A**) xylose, (**B**) butanol, (**C**) acetone, (**D**) ethanol, (**E**) butyric acid, (**F**) acetic acid and (**G**) biomass in g L^{-1} with *C. acetobutylicum* with pure xylose in batch mode.

Based on the xylose results, fermentation with mixtures of glucose/xylose (final monosaccharide concentration: 60 g L^{-1}) were developed for two sugar compositions. A 1:1 sugar ratio was first used (Figure 3). The pH evolution can be found in Figure S1B. Biomass concentrations were about 60% lower than those obtained with pure xylose, resulting in values of 2.5–3.2 g L^{-1}, attributed to the lower biomass yield linked to glucose consumption. Capilla et al. [17] estimated a maximum biomass concentration of about 3 g L^{-1} with 33 g L^{-1} of pure glucose. The case of using calcium carbonate and a pH control strategy had a notably different behavior. This exhibited diauxic growth linked with

the xylose uptake from hour 48 onwards after 24 h of pure glucose depletion (Figure 3A,B). Butyric acid production linked to the consumption of both substrates also occurred using calcium carbonate and the pH minimum of 5.1, first at a rate of 0.52 g L^{-1} h^{-1} and then 0.18 g L^{-1} h^{-1} (Figure 3F).

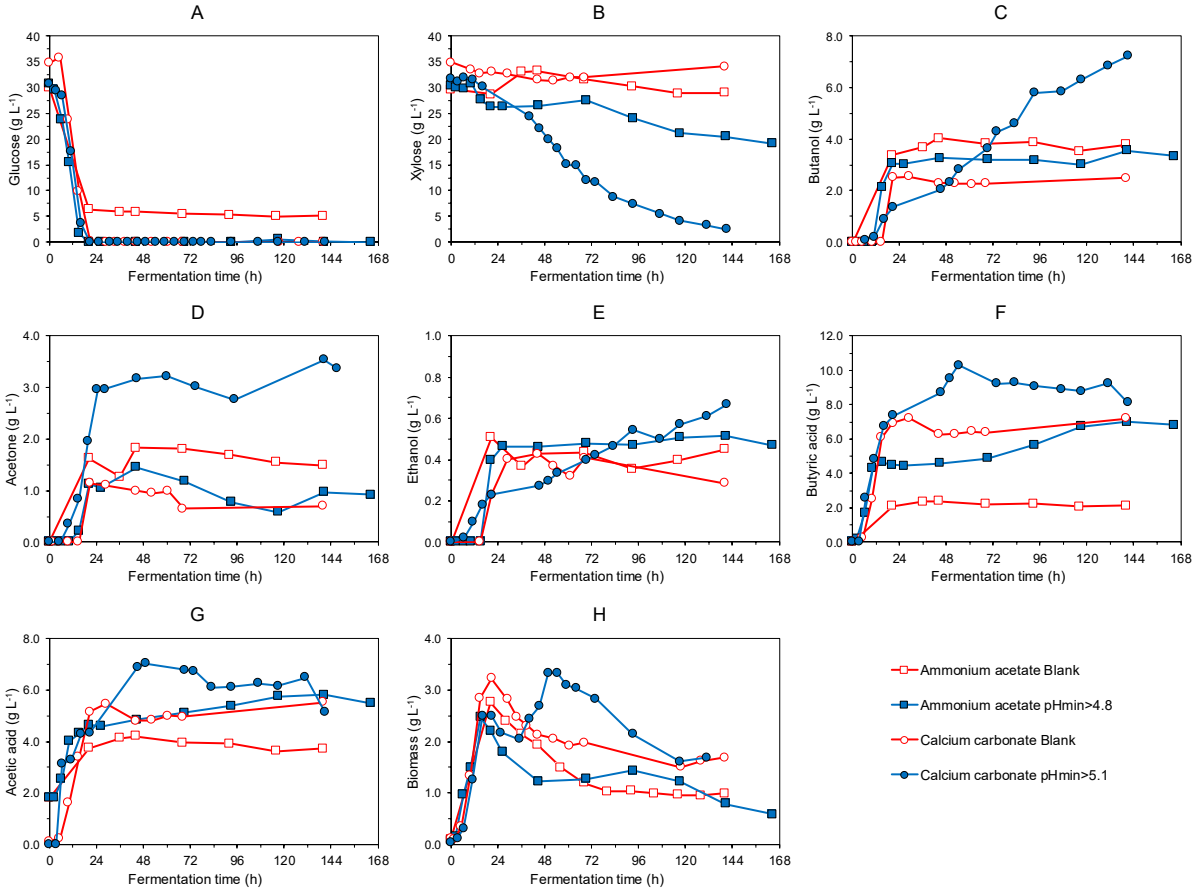

Figure 3. Fermentation profiles of (**A**) glucose, (**B**) xylose (**C**) butanol, (**D**) acetone, (**E**) ethanol, (**F**) butyric acid, (**G**) acetic acid and (**H**) biomass in g L^{-1} with *C. acetobutylicum* with 1:1 ratio of glucose/xylose in batch mode.

This second butyric production resulted in a peak of 10.3 g L^{-1} of butyric acid, similar to the maximum concentration achieved with pure xylose (Figure 2E). As can be seen, this is the only operational condition in which xylose uptake was concomitant to butanol production (7.2 g L^{-1}) (Figure 3B). The beneficial effect of calcium carbonate as a buffer regulator was also found by Kanouni et al. [24]. Otherwise, a pH of 4.8 does not produce good xylose uptake when ammonium acetate is the buffer, so that the buffer regulation itself has an impact on xylose uptake for a specific mixture of glucose and xylose. This highlights the importance of a tailor-made strategy not only for pH conditions but also the type of buffer. These results show the potential of *C. acetobutylicum* for biobutanol production when sugar mixtures of glucose and xylose are employed, which could be the case of lignocellulosic substrates, among others.

To mimic the conditions of lignocellulosic hydrolysates, a glucose/xylose ratio of 3:1 (final monosaccharide concentration: 60 g L^{-1}) was also tested, and the same conditions as with previous experiments were evaluated. Calcium carbonate was tested at two pH levels of 4.8 and 5.1 to elucidate the impact of the control of different minimum pH levels. The pH profiles are shown in Figure S1C. A similar biomass production was obtained by comparing it with the 1:1 glucose/xylose mixture at a maximum concentration of about 2.7–2.9 g L^{-1} for the non-pH-controlled reactors and pH-controlled reactor with calcium carbonate. A higher biomass concentration was achieved with ammonium acetate and

pH control (Figure 4H). Reducing the xylose level and increasing the glucose, no diauxic growth occurred in the pH-controlled reactor buffered with calcium carbonate at either pH 5.1 or 4.8. Glucose consumption stopped either by the end of the fermentation due to acid crash (blank) or glucose exhaustion (pH $\geq$ 4.8–5.1) with both buffers (Figure 4A). Unlike ratio 1:1, calcium carbonate fermentation with either active pH control (pH $\geq$ 4.8–5.1) did not show any xylose uptake after glucose depletion with a sugar ratio of 3:1. As the failure of xylose metabolisms with calcium carbonate as buffer reagent could be attributed to the low xylose concentration on the media (15 g L^{-1}), it seems that a minimum concentration of xylose is required to modulate the pathway to xylose by *C. acetobutylicum*.

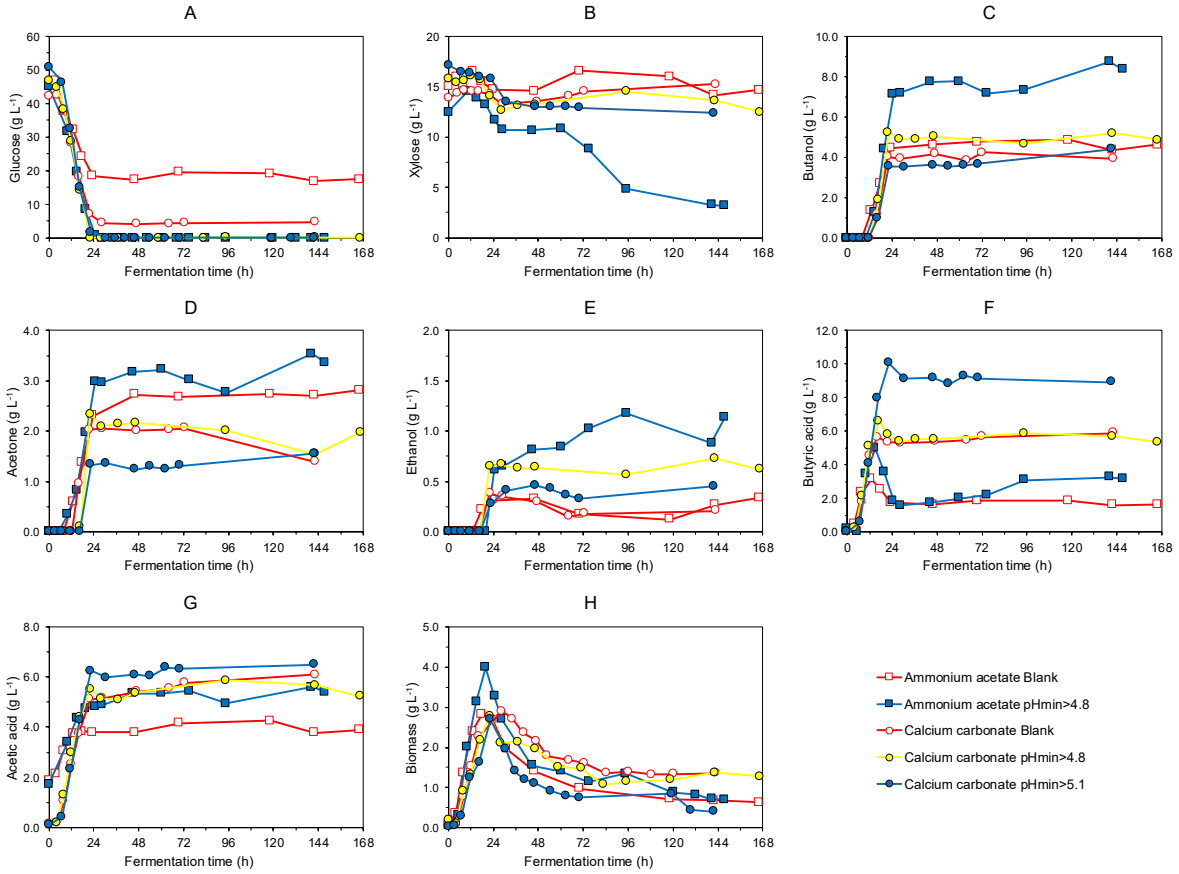

Figure 4. Fermentation profiles of (**A**) glucose, (**B**) xylose (**C**) butanol, (**D**) acetone, (**E**) ethanol, (**F**) butyric acid, (**G**) acetic acid and (**H**) biomass in g L^{-1} with *C. acetobutylicum* with 3:1 glucose/xylose ratio in batch mode.

In contrast, the use of ammonium acetate instead of calcium carbonate promoted xylose depletion, although the xylose uptake started later (72 h and 48 h after glucose depletion) than in the previous experiment, which also shows that low xylose concentrations complicate the metabolic shift from glucose to xylose consumption. Indeed, there was no biomass growth associated with xylose consumption, as when using 30 g L^{-1} of xylose. The monosaccharide would therefore be used for cell maintenance rather than biomass production. The lower effectiveness of pH-controlling strategies on xylose uptake at higher ratios of glucose/xylose was also found by Jiang et al. [13]. For the experiments without noticeable xylose uptake, butanol (ABE) production ranged from 3.6–4.9 (5.2–8.2) g L^{-1}, which is in agreement with the higher glucose from a prior experiment (2.6–4 g L^{-1} butanol production with 30 g L^{-1} of glucose). The ammonium acetate with pH $\geq$ 4.8 experiment successfully consumed nearly all the xylose, and maximum butanol (ABE) production was 8.8 (13.2) g L^{-1}. This concentration was very similar regardless of sugar composition when 60 g L^{-1} of either xylose or xylose and glucose was used, although the type of buffer

regulation and pH control parameters were different for each case. This was related to the butanol concentration, as 8–8.5 g L^{-1} has been shown to be inhibitory when *C. acetobutylicum* is grown with xylose [11,23]. The best condition at ratio 3:1 was a pH control at 4.8 and ammonium acetate as the buffer reagent. In these conditions, maximum butanol (ABE) productivity was 0.61 g L^{-1} h^{-1} (0.89 g L^{-1} h^{-1}). Fermentation in continuous mode was also tested to improve butanol productivity.

3.2. Continous Fermentation

The pH regulation strategy was evaluated in a continuous fermentation filled with plastic rings to operate at high D values while preventing biomass washout. To promote biofilm growth, the pH was first kept at 6.0, after which pH was shifted to the minimum of 4.8 to promote solvent production. During the biofilm formation stage, it was fed 60 g L^{-1} of glucose with a pH $\geq$ 6.0 (acidogenic optimum pH) at a dilution factor of 0.042 h^{-1} (Figure 5). The biomass was seen to grow on biofilm interlacing with the plastic rings. After 72 h (2 residence time (θ) from the start of continuous fermentation), the biomass concentration leaked less than 3 g L^{-1}, indicating successful biomass retention. At this time, the dilution factor was doubled to 0.083 h^{-1}, with a transitory biomass leak (96 h). Thereafter, the suspended biomass remained stable for the rest of the experiment at 3.9 $\pm$ 0.9 g L^{-1}, indicating no washout on the duplication of the dilution factor until the end of the experiment (maximum dilution rate 0.333 h^{-1}). This configuration allowed a fast start-up and high biomass retention. At 120 h, the dilution factor was again doubled to 0.167 h^{-1}. As solventogenesis was inhibited in this pH, the conditions were very low solvent production and high acid concentrations. At 144 h (D = 0.167 h^{-1}), pH was allowed to drop to the solventogenesis set point (pH $\geq$ 4.8). In less than 24 h, the acids were consumed while butanol (ABE) was produced. In steady state conditions, a butanol (ABE) concentration of 9.0 $\pm$ 2.0 (10.1 $\pm$ 1.1) g L^{-1} (productivity: 1.5 $\pm$ 0.3 (1.7 $\pm$ 0.2) g L^{-1} h^{-1}) was achieved, indicating proper transition to solventogenesis.

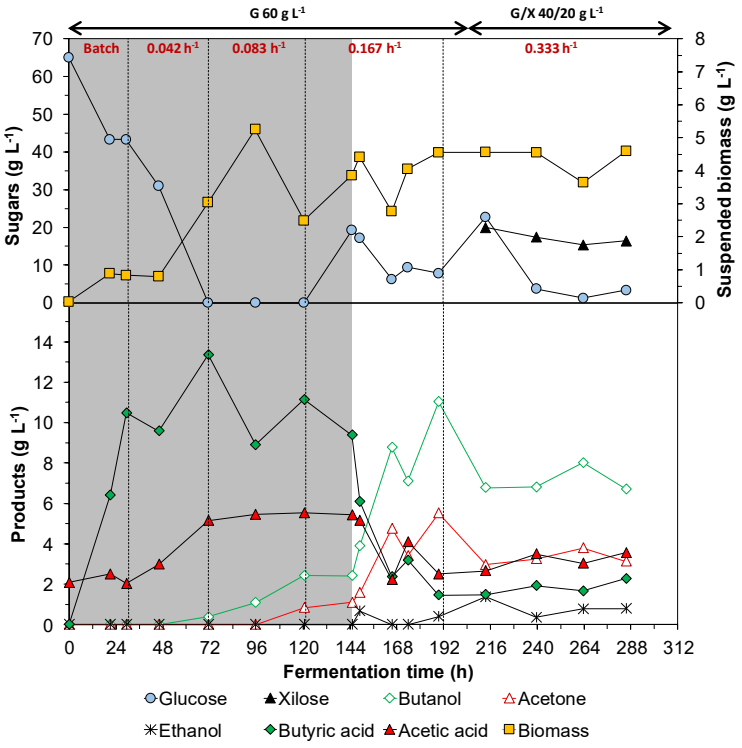

Figure 5. Fermentation profile of *C. acetobutylicum* with plastic rings at different dilution rates (h^{-1}) and monosaccharide feed. Grey area shows acidogenic phase (pH $\geq$ 6.0) while unshaded area is solventogenic phase (pH $\geq$ 4.8). G denotes glucose and X xylose.

The butanol (ABE) concentration achieved here was close to the maximum reported in batch reactors with the same glucose concentration and similar pH modulation strategy [17]. At 192 h, the dilution factor was again doubled to 0.333 h^{-1}. After 8 θ, steady state butanol values (ABE) were 6.8 (11.1) g L^{-1} with a productivity of 2.3 (3.7) g L^{-1} h^{-1}. At 216 h, glucose input was thus changed to a glucose/xylose mixture (2:1 ratio) to test the effect of varying the monosaccharide concentration, after which the butanol concentration was kept stable for at least 24 θ, with an average butanol (ABE) concentration of 7.1 ± 0.6 (11.2 ± 1.0) g L^{-1}. This concentration led to a butanol (ABE) productivity of 2.4 ± 0.2 (3.7 ± 0.3) g L^{-1} h^{-1} with a 2:1 glucose/xylose mixture. Additionally, the mass balance analysis is presented in Table 3. As expected, the distribution of the products manifested opposite trends depending on the pH set point. Butyric acid was the main product at pH ≥ 6.0, while butanol was the major product at pH ≥ 4.8. Moreover, the unaccounted yield would be associated to biofilm formation and CO_2 production. In this sense, previous authors reported that in ABE fermentation, the CO_2 could be up to a third of the metabolites produced [25].

Table 3. Mass balance analysis for the continuous experiment.

Time (h)	Substrate (g L^{-1})	pH	*D* (h^{-1})	g C g C of Monosaccharide Consumed^{-1}							
				Acetic Acid	Butyric Acid	Butanol	Acetone	Ethanol	Suspended Biomass	Yield	Unaccounted
0–30	Glucose (60)	6.0	Batch	0.00	0.66	0.00	0.00	0.00	0.05	0.70	0.30
30–72	Glucose (60)	6.0	0.042	0.14	0.39	0.01	0.00	0.00	0.05	0.59	0.41
72–121	Glucose (60)	6.0	0.083	0.09	0.21	0.06	0.02	0.00	0.09	0.47	0.53
121–144	Glucose (60)	6.0	0.167	0.13	0.30	0.10	0.04	0.00	0.13	0.70	0.30
144–192	Glucose (60)	4.8	0.167	0.07	0.05	0.28	0.13	0.01	0.11	0.65	0.35
192–216	Glucose (60)	4.8	0.333	0.07	0.05	0.27	0.11	0.05	0.16	0.72	0.28
216–288	Glucose/Xylose (40:20)	4.8	0.333	0.08	0.07	0.28	0.13	0.02	0.14	0.72	0.28

The productivity obtained with a 2:1 glucose/xylose mixture (D = 0.333 h^{-1}) is almost four times more than that obtained in the batch reactor at a ratio of 3:1 with ammonium acetate and pH ≥ 4.8 (butanol (ABE) productivity of 0.61 (0.89) g L^{-1} h^{-1}). Using sugarcane bagasse for biomass immobilization, solvent productivities in the range of 1.8–2.1 g L^{-1} h^{-1} at D ranging 0.3–0.6 h^{-1} have been obtained [26]. In continuous operation mode at dilution factors ≥ 0.167 h^{-1}, glucose uptake was about 85% when 60 g L^{-1} was fed, while full consumption was at 40 g L^{-1} and xylose uptake was only 18 ± 8%. The lower xylose uptake than the batch reactor (74%) can be related to the continuous availability of glucose, which would mostly inhibit extending the shift of the metabolism to xylose. This shows the feasibility of developing an efficient continuous reactor configuration avoiding biomass washout and obtaining high stable productivities. However, further investigation is required to enhance the use of xylose in continuous operations.

4. Conclusions

The glucose/xylose ratio has a direct impact on the selection of the proper pH modulation strategy (pH control and/or buffer composition) in ABE fermentation using *C. acetobutylicum* DSM 792. Tailor-made pH modulation strategies should be developed for specific glucose/xylose mixtures to use the substrate. For a mixture of pure xylose (0:1), both buffers tested (calcium carbonate/ammonium acetate) led to similar results in terms of butanol production. However, minimum pH control worsened the solvent production. In the case of 1:1 glucose/xylose mixtures, calcium carbonate as a buffer reagent combined with the strategy of limiting minimum pH to 5.1 gave the best results, achieving nearly full consumption of xylose while producing close to 8 g L^{-1} of butanol. When the glucose/xylose ratio was raised to 3:1, the buffer ammonium acetate with pH ≥ 4.8 performed

better, achieving a butanol concentration of 8.8 g L^{-1} and almost complete xylose uptake. The continuous reactor configuration filled with plastic rings for biomass retention successfully increased butanol (ABE) productivity while preventing biomass washout. This configuration led to a biobutanol productivity of 2.4 ± 0.2 g L^{-1} h^{-1} at D = 0.333 h^{-1} with a sugar mixture of 2:1, which is almost four times greater than that obtained in batch operations. However, in this configuration xylose consumption worsened to 18 ± 8%. Further studies should thus be carried out to improve substrate use in continuous mode.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8050226/s1, Figure S1, The pH profiles of *C. acetobutylicum* under different carbon sources (A) pure xylose, (B) ratio 1:1 (C) ratio 3:1 in batch mode.

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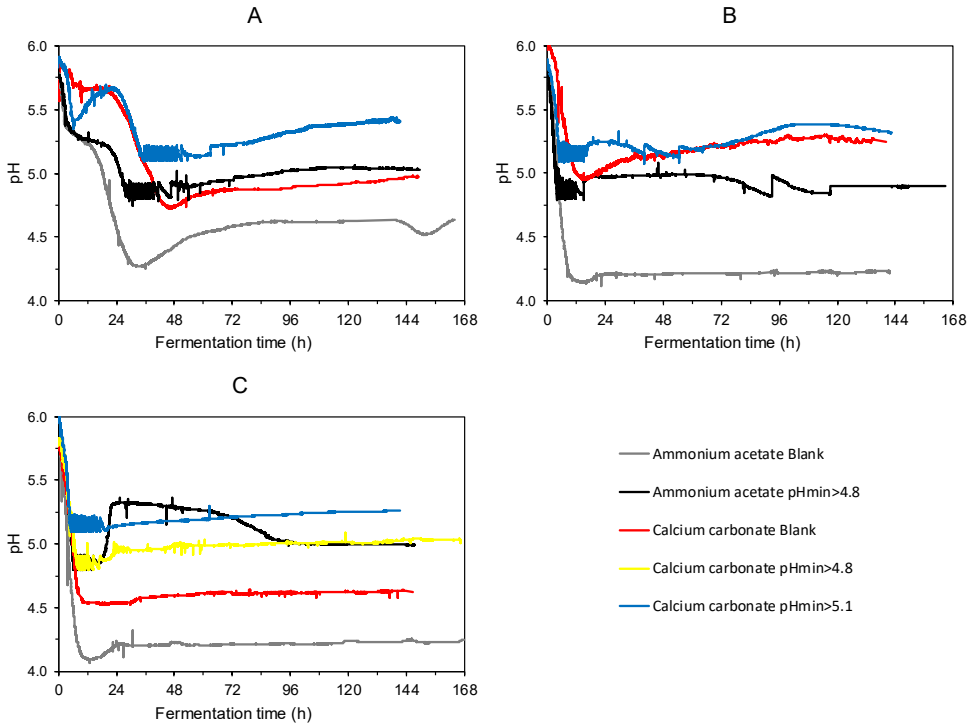

Figure S1. The pH profiles of *C. acetobutylicum* under different carbon sources (A) pure xylose, (B) ratio 1:1 (C) ratio 3:1 in batch mode.

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ORIGINAL ARTICLE

Solvent production from rice straw by a co-culture of *Clostridium acetobutylicum* and *Saccharomyces cerevisiae*: effect of pH control

Miguel Capilla[1] · Alejo Valles[1] · Pau San-Valero[1] · Francisco Javier Álvarez-Hornos[1] · Carmen Gabaldón[1]

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Abstract
One of the challenges in biofuel production from lignocellulosic wastes is to improve its conversion to solvents; therefore, new strategies to enhance xylose uptake are required due to be the secondary abundant sugar. In this context, a novel fermentation strategy integrating a co-culture of *Clostridium acetobutylicum* and *Saccharomyces cerevisiae* with pH control was developed. Initially, two different buffers, ammonium acetate and calcium carbonate, were tested under $pH_{min} > 4.8$ by fermenting 60 g L^{-1} of glucose with the *C. acetobutylicum* monoculture. Ammonium acetate was selected for fermenting media as butanol production was increased from 9.8 to 10.9 g L^{-1} over the calcium carbonate test. Comparing with the spontaneous acetone-butanol-ethanol (ABE) fermentation with *C. acetobutylicum* when no xylose consumption was observed, xylose consumption was efficiently increased by controlling $pH_{min} > 4.8$. The xylose consumption was > 47% either by using a 45:15 g L^{-1} glucose:xylose mixture or with rice straw (RS) hydrolysate. *Clostridium* monoculture using RS hydrolysate and $pH_{min} > 4.8$ produced a butanol (ABE) concentration of 6.5 (9.5) g L^{-1}. While it increased to 7.0 (13.1) g L^{-1} when the co-culture with *S. cerevisiae* was used using same pH regulation strategy mainly due to ethanol increase up to 2.7 g L^{-1}. Moreover, the xylose uptake doubled to 94% due to amino-acid secretion by yeast. Overall, this combined strategy was a very effective method for promoting sugar consumption and ABE solvent production from lignocellulosic waste.

Keywords ABE fermentation · Ethanol fermentation · Lignocellulosic waste, pH control · Xylose

1 Introduction

The world is moving towards a more sustainable economy based on the use of renewable sources urged, among other factors, by the climate change awareness and the necessity of limiting the greenhouse gas emission [1]. Butanol is a highly appreciated biofuel due to its physicochemical properties and would not require extensive investments as it can be delivered via current infrastructure [2–4]. Biobutanol can be produced via acetone-butanol-ethanol (ABE) fermentation by solventogenic *Clostridia*. However, ABE fermentation has downsides as low concentrations or substrate costs. First-generation biobutanol was produced by sugar- or starch-based feedstock (like sugarcane or corn) with drawbacks as high cost of raw materials and competition in the food supply. Thus, the lignocellulosic biomasses from agro-food activities have grown in interest due to low cost and wide availability [5]. Lignocellulosic biomasses, such as rice straw (RS), require a pretreatment prior to sugar release from cellulose and hemicellulose after enzymatic hydrolysis. Among the pretreatments, alkalis are capable of swelling the cellulose structure and remove acetyl groups, lignin and uronic acid substitutions, hence increasing enzyme accessibility to the polysaccharides [6]. From a compendium of 77 hydrolyzed lignocellulosic feedstock, Birgen et al. [7] obtained a median value of 23.6 and 10.8 g L^{-1} of glucose and xylose (glucose:xylose ratio of ~ 2.2:1); thus, xylose is the secondary monosaccharide in hydrolysate composition. However, the carbon catabolite repression (CCR) of glucose over xylose hinders the overall efficiency of ABE fermentation [8], being one of the drawbacks when using lignocellulosic biomass.

Regarding solventogenic *Clostridium* species, it was reported that *Clostridium beijerinckii* is capable of uptake xylose efficiently in presence of glucose [9], which could be

✉ Pau San-Valero
pau.valero@uv.es

[1] Research Group in Environmental Engineering (GI2AM), Department of Chemical Engineering, University of Valencia, Av. De la Universitat S/N, 46100 Burjassot, Spain

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due to the presence a big gene cluster of D-xylose pathway genes found in some strains such as *C. beijerinckii* NCIMB 8052 [10]. In the case of *Clostridium acetobutylicum*, previous studies reported that the xylose catabolic routes, pentose phosphate [11] or the phosphoketolase pathway, are influenced by the level of xylose present in the media [12]. Thus, more efforts should be made to elucidate strategies to better control the xylose uptake. This is especially important in the case of glucose:xylose mixtures to avoid CCR, among the singular characteristics which make the study of *C. acetobutylicum* of interest highlights its capability to form a denser biofilm due to cell to cell communication [13], which could led to the increase the overall productivity by promoting cell immobilization [14].

In addition, the media pH has been shown to play an important role on the fermentation profile of *C. acetobutylicum*. For example, controlling the minimum pH on batch fermentation boosted glucose consumption and butanol production by alleviating acid crash, with an increment from 7.47 to 11.22 g L^{-1} butanol when comparing with no pH control [15]. Regulation of pH was also successfully implemented in continuous reactors with *C. acetobutylicum* using lignocellulosic substrates [16]. Moreover, pH-controlled continuous fermentations have been also shown effective with xylose [17, 18]. Another strategy that has been shown efficient to improve sugar uptake and solvent production by *C. acetobutylicum* was formulation of the media with $CaCO_3$ producing 9.65 g L^{-1} of butanol from a glucose:xylose mixture (30:30 g L^{-1}) with 82% of sugar consumption [19]. Indeed, $CaCO_3$ was demonstrated to reduce the residual xylose with glucose:xylose mixtures regardless of the monosaccharide ratio in ABE fermentation [20]. Jiang et al. [21] also demonstrated that pH control could increment xylose uptake despite their sugar ratio. Best results were achieved at the lower glucose:xylose ratio tested (1.5:1); consumption of xylose was enhanced from 11.6% without pH control to 66.1% by controlling pH after the acidogenic phase. Therefore, it seems that pH regulation independently of the implemented strategy (control pH or media buffering) could improve the xylose consumption in presence of glucose.

As a considerable fermentation strategy, co-culture of two or more microorganisms has been applied in several bioprocesses to confront the limitations of pure strains, like biofuels or food industries [22]. Some examples are the consolidated bioprocessing (CBP) to combine cellulolytic bacteria and another microorganisms, such as lactic acid bacteria [23] or the co-culture with amylolytic and ethanol fermentation microorganisms to develop competitive simultaneous saccharification and fermentation (SSF) [24, 25]. Recently, co-culture of *S. cerevisiae* and *Clostridium* species have gained interest. The metabolic abilities of the yeast could contribute to increase monosaccharide uptake and biofuel production (butanol and ethanol) at the co-culture with *Clostridium* species. The better outcome of the fermentation may be due to the yeast ability to secrete amino acids to the fermentation media [26, 27]. Some studies have shown that co-culture of *S. cerevisiae* and *C. acetobutylicum* improves butanol production from starch-based media [27, 28], although its application to lignocellulosic waste remains unexplored. For instance, Qi et al. [29] were able to increase ABE production from 17.66 to 42.56 g L^{-1} by using a co-culture with *S. cerevisiae* and *C. acetobutylicum* CH02 over the *Clostridium* monoculture when 150 g L^{-1} of cassava was fermented.

The main aim of this work was to evaluate the combination of pH regulation and a co-culture system of *C. acetobutylicum* and *S. cerevisiae* with the target to improve solvent concentrations and sugar uptake from RS hydrolysate. Initially, it was evaluated the effect of the pH control in the exploitation of model substrates (glucose and xylose) by *C. acetobutylicum* for butanol production by using two buffering components (acetate and carbonate). The effect of the best buffering component was further tested with hydrolysates from alkaline-pretreated RS in order to define the pH regulation strategy. Once pH regulation was established, the co-culture system of *S. cerevisiae* and *C. acetobutylicum* was studied by using model substrates. The overall strategy was further validated by using alkaline-pretreated RS hydrolysates. This work is expected to contribute to enhance solvent concentrations and sugar consumption in fermentations from lignocellulosic biomass.

2 Material and methods

2.1 Microorganisms, fermentation media, and chemical reagents

C. acetobutylicum DSM 792 was purchased from DSMZ, Germany (German Collection of Microorganisms and Cell Cultures). The culture was stored in 20% glycerol at − 80 °C. Prior to the experiments, the microorganism was cultured statically at 37° in 19 g L^{-1} of Reinforced Clostridial Medium (RCM) fortified with 10 g L^{-1} of glucose as seed inoculum. *S. cerevisiae* EYS4 was maintained on YPD agar (yeast extract, 10 g L^{-1}, peptone 20 g L^{-1}, glucose 20 g L^{-1}, and agar 20 g L^{-1}) at 4 °C. The yeast seed culture was inoculated into 50 ml of YPD broth and incubated at 37 °C and 150 rpm. The fermentation medium composition was (g L^{-1}): sugars (glucose, xylose, or hydrolysate); yeast extract, 5; K_2HPO_4, 0.5; KH_2PO_4, 0.5; NH_4Cl, 2; $MgSO_4.7H_2O$, 0.2; $MnSO_4.7H_2O$, 0.01; $FeSO_4.7H_2O$, 0.05; resazurin sodium salt, 0.001; and antifoam 204, 0.01%. Two different media formulations were tested. Acetate buffer (CH_3COONH_4; 2.2 g L^{-1}) replaced the NH_4Cl of the fermentation medium. Carbonate buffer was formulated by adding $CaCO_3$ (2.5 g

L^{-1}) to the above-mentioned fermentation media. The media was sterilized by autoclave at 121 °C during 20 min and the metal solution was filter-sterilized by 0.22 µm. Chemicals were obtained from VWR, except for antifoam 204 (Sigma-Aldrich), $CaCO_3$ (Merck), and yeast extract (Alfa Aesar).

2.2 RS pretreatment and hydrolysis

The biomass was obtained from local farmers of L'Albufera situated close to Valencia (Spain). The untreated RS composition on dry weight basis was 35.8 ± 2.1% of cellulose, 17.5 ± 1.4% of hemicellulose, 0.1 ± 0.0% of acid soluble lignin, 14.3 ± 0.4% of acid insoluble lignin, 16.7 ± 0.1% of ash, and 15.6% of others. Prior optimized from a previous study [30], the following conditions were applied to obtain the hydrolysate: dried RS was milled to 0.1- to 2-mm particle size; it was pretreated with 0.75% NaOH and a solid loading of 5% (w/w) at 134 °C for 40 min in an autoclave (MED20, J.P. Selecta, Spain), then dried in an oven at 45 °C for 24 h prior storage at 4 °C. Enzymatic hydrolysis was performed with 8% (w/w) solids loading at pH 5.2 with a concentration of 20 FPU g-dw^{-1} (Cellic® CTec2, Novozyme, Denmark) at 50 °C and 150 rpm for 72 h in an orbital incubator (G25, New Brunswick Scientific, USA). The hydrolysate was stored at − 4 °C prior use.

2.3 Experimental setup

2.3.1 Monoculture and co-culture reactors

The reactor fermentations were performed with a working volume of 0.8 L (total volume of 1.1 L). The media was flushed with nitrogen gas for 30–35 min before inoculation. A 5% v/v inoculum of *C. acetobutylicum* was used in each experiment. The fermentations were performed at 37 °C and 120 rpm. For the co-culture experiments, a 5% v/v inoculum of *S. cerevisiae* was used. Two kinds of experiments without and with pH control were performed. A minimum set-point control was carried out with NaOH (3 M) to keep the pH above the threshold. The experiments were followed using a Tris-compatible flat pH sensor with LoggerPro software (Vernier, USA). The pH probes were sterilized following the procedure by Qureshi et al. [31]. They were sterilized by submerging them in a 50% ethanol solution (v/v) for 12–24 h. After which, the probes were washed with sterile water. Samples were taken at appropriate time points to analyze cell growth, sugars, and products (butyric acid, acetic acid, butanol, acetone, and ethanol).

2.3.2 Co-culture pre-screening

Fermentations were performed in 50-mL serum bottles with 40 mL of working volume; the inoculation was carried out with 5% v/v of *C. acetobutylicum* and then 5% v/v of *S. cerevisiae* at three time lags (0, 5, and 10 h). Monocultures of *C. acetobutylicum* and *S. cerevisiae* were performed as controls. Anaerobic conditions were obtained by sparging nitrogen in the fermentation medium. The fermentations were carried out in an orbital incubator at 37 °C and 150 rpm by duplicate. Butanol production was selected as the criteria for establishing the inoculation procedure of the co-culture reactors.

2.4 Experimental plan

Five sets (runs) of experiments were carried out to develop a combined regulation pH strategy with a co-culture fermentation of *C. acetobutylicum* and *S. cerevisiae*. All runs were performed without and with pH control. The value of the minimum pH (4.8) was selected from a prior study by using the same glucose concentration and 5 g L^{-1} carbonate [15]. Run 1 was performed with 60 g L^{-1} of glucose to determine the best buffer composition for the two formulations. From these results, ammonium acetate was selected as the best buffer alternative. For the rest of the experiments, two pH regulations were employed: (1) ammonium acetate dosage or (2) ammonium acetate dosage combined with minimum NaOH pH control. The effect of the pH regulation on xylose in presence of glucose was assessed (run 2). Run 3 was performed by replacing the synthetic substrate with alkali-pretreated RS as described in Sect. 2.2. In run 4, a model concept of co-culture was developed using a synthetic mixture of 35:15 glucose:xylose mimicking RS hydrolysate. In this case, *S. cerevisiae* was inoculated at a selected time after *C. acetobutylicum* according to results derived from Sect. 3.2.1. Once the co-culture model was established, the effect on solvent production was checked by using RS hydrolysate (run 5). Runs 3 to 5 were performed by duplicate.

2.5 Analytical methods

The fermentations were monitored by sampling at desired time points. Cell biomass was determined using an UV–Vis spectrophotometer (SpectroFlex 6600, WTW) at 600 nm (OD_{600}). Biomass concentration of *C. acetobutylicum* (gDM L^{-1}) was estimated by using gDM $L^{-1} = 0.2941 \cdot OD_{600} + 0.0331$ ($R^2 = 0.9908$). Samples were centrifuged at 10,000 rpm for 5 min (MEGA Star 3.0, VWR, Germany) and filtered by 0.22 µm for analysis. Sugars and products were determined by liquid chromatography (Agilent 1100 Series HPLC system, Agilent Technologies, USA) using a refractive index detector (RID) and diode array detector (DAD) with an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA). The system was operated at 50 °C. A refractive index detector (RID) was used to detect sugars, butanol, and ethanol, while

a diode array detector (DAD) was employed at 210 nm to detect acetic, butyric, and levulinic acids and at 280 nm to detect acetone, furfural, and 5-(hydroxymethyl)furfural (5-HMF). The mobile phase was 5 mM of sulfuric acid with a flow of 0.6 mL min^{-1}. The RID was kept at 35 °C. The running time of the analysis was 45 min. The Folin-Denis method was used to quantify the total phenolic compounds expressed as gallic acid equivalents [32]. The values of pK_a of acetic acid (4.76) and butyric acid (4.82) were used with the Henderson-Hasselbalch equation (Eq. (1)) to obtain the concentration of undissociated acids.

$$\mathrm{pH} = pK_a + \log\frac{[A^-]}{[HA]} \tag{1}$$

The glucose consumption rate was estimated at the exponential growth phase by Eq. (2):

$$-q_{\mathrm{glucose}} = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \tag{2}$$

where $-q_{\mathrm{glucose}}$ corresponds to the glucose consumption rate (g L^{-1} h^{-1}), S_{t1} and S_{t2} are the monosaccharide concentrations at the starting and ending point of the exponential growth phase (g L^{-1}), and t_2 and t_1 are the times at the beginning and end of the exponential growth phase (h).

3 Results and discussion

3.1 Solvent production from RS hydrolysate by *C. acetobutylicum*

3.1.1 Effect of the media buffer using glucose as model substrate

The influence of the buffer species on butanol production from glucose (60 g L^{-1}) as main substrate in RS hydrolysate was evaluated by using two alternative components, CH_3COONH_4 or $CaCO_3$, added to the buffering phosphate species (run 1). Main representative parameters of the ABE fermentation experiments without and with pH control ($pH_{min} > 4.8$) are summarized in Table 1. As it can be seen, the reactors without pH control showed a different behavior depending on the buffer compound. A butanol production of 11.2 g L^{-1} was achieved when acetate was used but only 3.2 g L^{-1} was obtained with carbonate. ABE production also differed, being 19.2 g L^{-1} with acetate and 5.9 g L^{-1} with carbonate. The substantial difference in solvent production with acetate compared with carbonate was related to the glucose consumption in both reactors. In that sense, the glucose was completely depleted (in less than 50 h) with acetate while the carbonate reactor only consumed 55% of the reducing sugar. Therefore, higher butanol yield was also observed (0.199 g g^{-1} for acetate and 0.093 g g^{-1} for carbonate). The pH recovery was higher for acetate (4.18 to 4.84) than for carbonate (4.57 to 4.71), which was connected to a better development of ABE fermentation when using acetate instead of carbonate. The nearly double glucose consumption rate with carbonate ($-q_{\mathrm{glucose}}$: 1.80 g L^{-1} h^{-1}) seemed to impact adversely on the solventogenesis. Indeed, the level of $CaCO_3$ (2.5 g L^{-1}) was not sufficient to prevent the acid crash phenomenon. The total undissociated acid species were higher than 60 mM, the referenced threshold for *Clostridium* species [33]. The lower performance of the reactor with carbonate over the reactor with acetate could be explained by the differences in the maximum butyric acid concentration. The production of acids can be modulated by changing the fermentation media; for example, calcium carbonate can enhance the production of acids in ABE fermentation. Ren et al. [8] observed an increment of ~ 1.3-fold when fermenting with P2 media over P2 media supplemented with calcium carbonate with *C. acetobutylicum* ATCC 824. Similarly, the reactor with acetate reached 2.87 g L^{-1} of butyric acid (Fig. 1a), while the reactor with carbonate reached 4.88 g L^{-1} (data not shown), thus showing an ~ 1.7-fold increase when the calcium carbonate is used to

Table 1 Solvent production, stoichiometric, and kinetic parameters under different buffer formulations with glucose (60 g L^{-1}) as carbon source

	Buffer	Butanol-$_{max}$ (g L^{-1})	ABE_{max} (g L^{-1})	$Y_{B/S}$ (g g^{-1})	$Y_{ABE/S}$ (g g^{-1})	Glucose conversion (%)	$-q_{\mathrm{glucose}}$ (g L^{-1} h^{-1})	pH_{min}	pH_{final}
No pH control	Ammonium acetate (2.2 g L^{-1})	11.2	19.2	0.199	0.334	100	0.96	4.18	4.84
	Calcium carbonate (2.5 g L^{-1})	3.2	5.8	0.093	0.172	55	1.80	4.57	4.71
pH control ($pH_{min} > 4.8$)	Ammonium acetate (2.2 g L^{-1})	10.9	16.2	0.181	0.269	100	2.67	4.80	5.58
	Calcium carbonate (2.5 g L^{-1})	9.8	15.1	0.172	0.264	100	1.90	4.80	5.33

Yields were estimated with the maximum solvent concentration. Glucose conversion was calculated at the end of the fermentation

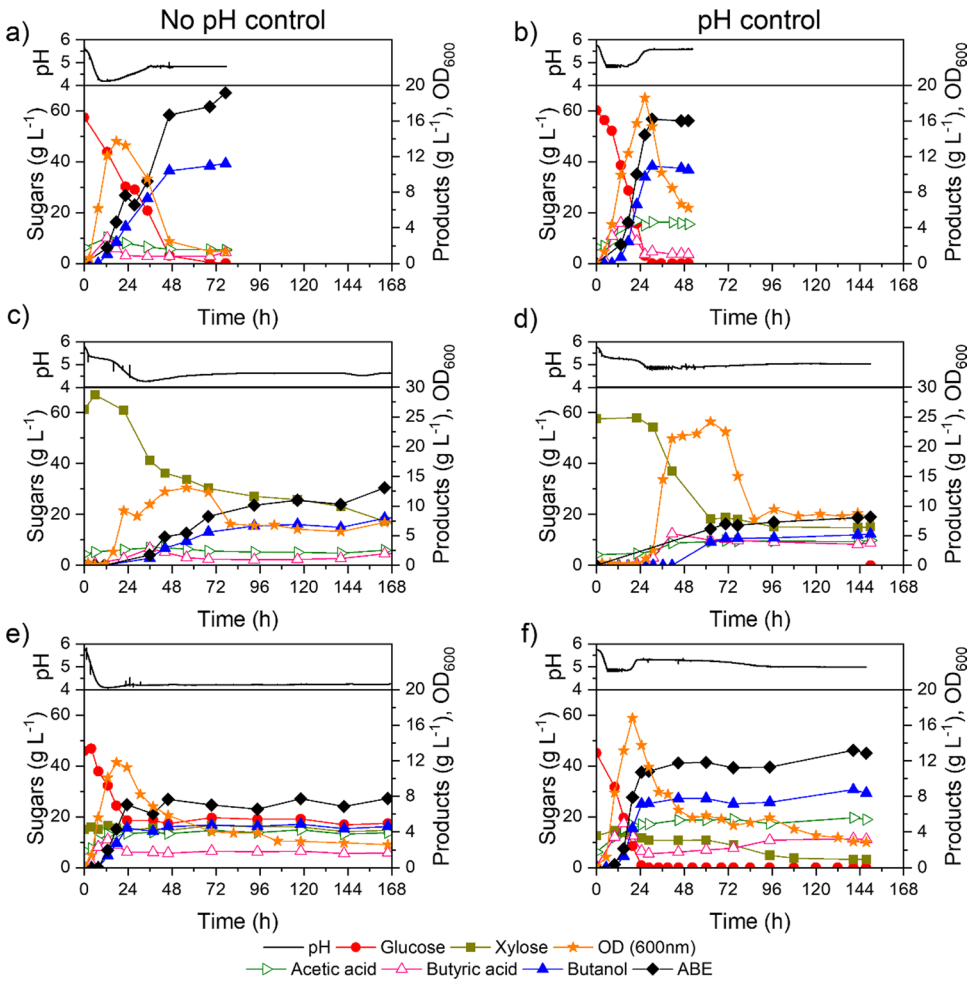

Fig. 1 ABE fermentation profiles by *C. acetobutylicum* DSM 792. Glucose (60 g L^{-1}): **a** no pH control and **b** pH control. Xylose (60 g L^{-1}): **c** no pH control and **d** pH control. Glucose:xylose (45:15 g L^{-1}): **e** no pH control and **f** pH control

buffer the fermentation. These results indicated the effect of buffer media formulation on *C. acetobutylicum* DSM 792 metabolism. In this way, Luo et al. [34] obtained 1.2 g L^{-1} of butanol when fermenting with 0.75 g of K_2HPO_4 and KH_2PO_4 as media buffer, showing that the usage of sole phosphate species as buffer failed to produce enough butanol. Higher level of carbonate (5 g L^{-1}) allowed to achieve better glucose consumption without pH control [15, 19], although acid crash was reported in some extent [15].

The pH regulation had an impact on the switch between acidogenesis and solventogenesis of *C. acetobutylicum* DSM 792 independently of the media buffer. For the two assays, the pH regulation was activated at early times (6–7 h) when acids production occurred, thus allowing a good pH recovery in both reactors after exponential growth phase ended, with final pHs > 5.3. Respecting the reactor with the acetate buffer, the final butanol concentration was very similar to the uncontrolled counterpart (10.9 over 11.2 g L^{-1}) despite the reduction in ABE solvents (16.2 over 19.2 g L^{-1}). In this case, ABE composition shifted to lower acetone proportion (6.1:3.2:0.7 butanol:acetone:ethanol), closer to the theoretical ratio (6:3:1), compared with the non-pH control reactors (acetate: 5.1:3.8:1.0; carbonate: 4.6:3.7:1.7). Furthermore, butanol yields were similar (> 0.18 g g^{-1}), showing that no butanol inhibition occurred when using acetate buffer without or with minimum pH control. Interestingly, the glucose consumption rate with ammonium acetate increased in ~ 2.8-fold with pH regulation (2.67 g L^{-1} h^{-1}), being the highest achieved over all the experiments. The fast sugar consumption rate shortened the fermentation time from ~ 50 to ~ 30 h. Therefore, a substantially increase in butanol productivity was achieved. The pH control enhanced glucose consumption rate with other *Clostridium* species such as *C. beijerinckii* IB4. A higher glucose consumption rate (1.67 ± 0.05 g L^{-1} h^{-1}) was achieved when pH was controlled (pH at 5.5 after reached) versus no pH control (1.03 ± 0.04 g L^{-1} h^{-1}) [35]. In the case of using carbonate, the pH regulation impacted favorably on the solvent production and glucose depletion, but not in the glucose consumption rate. The complete depletion of glucose led to an increment in butanol (ABE) production from 3.2 (5.9) to 9.8 (15.1) g L^{-1} (Table 1), while butanol yield (0.172 g g^{-1}) was similar to that of the fermenter with acetate buffer. Furthermore, pH regulation at early stages was shown as a very

efficient strategy to avoid acid crash comparing with making changes on media formulation. Moreover, Jiang et al. [35] observed that delaying the pH control more over 24 h of the fermentation did not avoid acid crash. Our approach, based on fixing a minimum pH rather than establishing a specific time to start pH control, allows well-fitting the pH regulation during the exponential growth phase independently of the growth kinetics. In our case, both buffers exhibited good performance in solvent production when active pH control was used. The main difference between them was in the glucose rate, being 40% higher in the case of using acetate. Consequently, the higher glucose consumption rate with ammonium acetate led to a greater productivity over the use of calcium carbonate while reducing the fermentation time. Moreover, without pH regulation the use of 2.2 g L^{-1} of ammonium acetate does not exhibit acid crash leading to better solvent production than 2.5 g L^{-1} of calcium carbonate. Thus, by the criteria of incrementing the solvent productivity (with pH regulation) or overcome acid crash (without pH regulation), subsequent experiments were carried out with ammonium acetate as media buffer.

3.1.2 Effect of the pH regulation on xylose and mixture of glucose:xylose

In order to evaluate the exploitation of lignocellulosic wastes onto ABE solvents, the effect of the pH regulation on xylose (secondary reducing sugar) consumption under the presence of glucose (primary reducing sugar) was studied. Two sets of experiments were performed by keeping constant the reducing sugar concentration at 60 g L^{-1} and in absence or presence of glucose (run 2). The selected mixture was of 45:15 g L^{-1} glucose:xylose, which is on the typical proportion between the two monosaccharides in hydrolysates from lignocellulosic waste. Fermentation profiles with uncontrolled pH and controlled pH using xylose and with a mixture of glucose:xylose were compiled in Fig. 1c-f. For comparison purposes, patterns with pure glucose and same media formulation (2.2 g L^{-1} acetate; Table 1) were also depicted (Fig. 1a, b). From the reactors with pure xylose (Fig. 1c, d), it can be seen a lag phase of approximately 24 h not observed previously with glucose (Fig. 1a, b). This is linked to the change of the carbon source from the inoculum growth (RCM with glucose). Without pH control, similar sugar consumption rates were achieved during exponential growth phase, independently of the monosaccharide (glucose: 0.96; xylose: 1.13 g L^{-1} h^{-1}). Nevertheless, xylose consumption declined from 48 h when butanol concentration started to increase (Fig. 1c) while no changes on consumption were observed with glucose. This reduction in xylose uptake would be related to product inhibition. In this sense, a butanol concentration of ~ 8.0 g L^{-1} had been reported as inhibitory when using xylose as carbon source [36]. At the end of the fermentation, only 72% of xylose was consumed, whereas butanol production reached the inhibitory value (8.0 g L^{-1}; butanol yield of 0.181 g g^{-1}). On the other hand, the fermentation of the sugar mixture (45:15) without pH control stopped at 24 h due to an acid crash phenomenon (Fig. 1e). Nevertheless, a butanol (ABE) production of 4.9 (7.7) g L^{-1} was achieved from a glucose consumption of 63%, with a butanol (ABE) yield of 0.182 (0.289) g g^{-1}. Glucose consumption rate, with a 25% lower initial glucose level, was faster in the mixture (1.35 g L^{-1} h^{-1}) than with pure glucose (0.96 g L^{-1} h^{-1}), however was not sufficiently high to promote the solventogenesis shift before acid crash occurred. Results indicate that a minimum level of initial glucose would be required for completion of ABE fermentation with this strain when pH evolved spontaneously.

A similar xylose consumption rate was observed with and without pH control; however, the pH control caused a carbon flux redistribution to boost biomass production (Fig. 1d). A OD_{600} ~ 1.6-fold higher was achieved (at the maximum point) when using pure xylose comparing with the use of glucose as carbon source (xylose: 24.2 or 7.2 g L^{-1} at ~ 62 h; glucose: 14.9 or 4.4 g L^{-1} at ~ 26 h). We speculate that the metabolism shift towards cell synthesis was associated to the availability of organic nitrogen from yeast extract (5 g L^{-1}). W. Jiang et al. [21] observed some increase in biomass growth with *C. acetobutylicum* ATCC 824 under pH control after the acidogenic phase with same yeast extract concentration, which was accompanied by an improvement of solvent production. However, contrarily to them we observed a decrease in butanol (ABE) production from 8.0 (13.1) g L^{-1} without pH control to 5.3 (8.2) g L^{-1} with pH control at 4.8 (Fig. 1c, d), probably due to the excessive biomass growth achieved in our study. In any case, data have shown that xylose will be consumed after glucose exhaustion when using lignocellulosic hydrolysates. In the sugar mixture experiment, 60 h was required to start xylose consumption after glucose depletion. In this regard, the utilization of pH control with the glucose:xylose mixture allowed avoiding acid crash, thus enhancing the full glucose conversion in < 24 h ($-q_{glucose}$: 2.38 g L^{-1} h^{-1}), and favoring the further xylose uptake without an excessive biomass growth (Fig. 1f). Although the pH regulation was unable to avoid the CCR phenomena, positively this delay on xylose consumption did not adversely impact on the butanol (ABE) production, which was 8.8 (13.2) g L^{-1}. Indeed, solvent production was quite close to the estimated values from the experimental yields with solely monosaccharides under pH control (butanol: 9.3; ABE: 13.9 g L^{-1}). As conclusion, ABE fermentation with *C. acetobutylicum* DSM 792 benefits for pH control to efficiently produced ABE solvents from glucose and xylose mixtures. Moreover, the implementation of in-situ product recovery techniques would improve even

more the xylose uptake in presence of glucose by avoiding butanol inhibitory levels.

3.1.3 The pH validation strategy using RS hydrolysates

The pH regulation strategy was validated by replacing the model substrates by the alkali-pretreated RS hydrolysate (run 3; Fig. 2). The hydrolysate characteristics at the final medium can be observed in Table 2. At the beginning of the fermentation reducing sugar concentration was 34.0 ± 0.7 g L^{-1} of glucose and 14.0 ± 0.6 g L^{-1} of xylose. In addition, the initial acetic acid was 4.9 ± 0.7 g L^{-1}. The cellobiose and arabinose remained nearly unchanged through the experiment. The total concentration of phenolic compounds was 0.13 ± 0.01 g L^{-1}. It was expected from the assay with the mixture model (Fig. 1e) that the lack of pH control caused an acid crash stopping the fermentation at 24 h (Fig. 2a), hence corroborating that low initial levels of glucose require of pH control for ABE fermentation with this strain. Under pH control, a butanol (ABE) production of 6.5 ± 0.1 (9.5 ± 0.8) g L^{-1} was achieved at the end of the fermentation. The pH control favored the consumption of xylose after glucose was depleted (47 ± 10%). Some xylose remaining as final butanol approached to inhibitory levels. In addition, butanol and ABE yields (butanol: 0.167 ± 0.005, ABE: 0.261 ± 0.002 g g^{-1}) were slightly better over the synthetic mixture (butanol: 0.161, ABE: 0.243 g g^{-1}). The complex composition of the RS hydrolysate better employed the sugar content and mitigated the CCR phenomena when the pH control was applied. In fact, the butanol (ABE) predicted concentrations from the results obtained with the synthetic media were 7.0 (10.5) g L^{-1}, similar to the experimental productions obtained in the assay (6.5 (9.5) g L^{-1}), thus validating the pH control strategy in terms of solvent production. The glucose consumption rates were 0.96 ± 0.13 and 2.38 ± 0.02 g L^{-1} h^{-1} for no pH and pH-controlled fermentations, respectively. Similar values were obtained with synthetic (pure glucose and sugar mixture). Hence, demonstrating no inhibition of glucose uptake occurred when the RS hydrolysate is fermented. The production herein obtained are among the referenced concentrations. For example, Amiri et al. [37] produced 7.1 (10.5) g L^{-1} of butanol (ABE) by using organosolv-pretreated RS with *C. acetobutylicum*

Table 2 Hydrolysate characteristics in the fermentation medium

Compound	Concentration (g L^{-1})
Glucose	34.0 ± 0.7
Xylose	13.0 ± 0.6
Cellobiose	4.5 ± 0.2
Arabinose	1.5 ± 0.1
Acetic acid	4.9 ± 0.7
Phenolic compounds	0.13 ± 0.01
Levulinic acid	N/D
Furfural	N/D
5-HMF	N/D

N/D denotes not detected

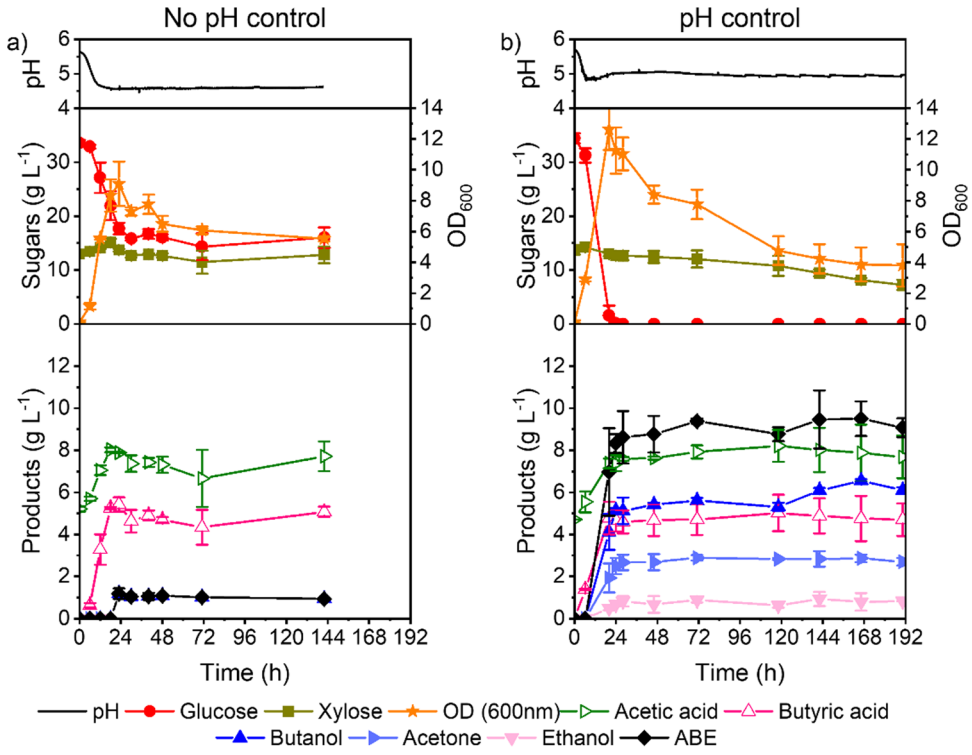

Fig. 2 ABE fermentation profiles by *C. acetobutylicum* DSM 792 with RS hydrolysate. **a** no pH control and **b** pH control

NRRL B-591. Other *Clostridium* species can be also used; in this sense, Valles et al. [30] achieved a higher butanol (ABE) concentration of 10.1 (16.7) g L^{-1} with alkali-pretreated RS using *C. beijerinckii* DSM 6422.

3.2 Solvent production by co-culture of *C. acetobutylicum* and *S. cerevisiae*

3.2.1 Effect of time inoculation

Prior to evaluation of the effect of pH regulation on the co-culture of *C. acetobutylicum* DSM 792 and *S. cerevisiae* EYS4, it was established the elapse time between inoculations of both species to promote butanol production. A screening experiment with 50-mL serum bottles was carried out by inoculating *S. cerevisiae* at 0, 5, and 10 h after *C. acetobutylicum* inoculation. Initial glucose and xylose levels mimicked the RS hydrolysate concentration (35 and 15 g L^{-1} of glucose and xylose). To analyze the effect of inoculation time on the substrate competition between the two species, and hence on the solvent redistribution, solvent production at early stage (24 h) was considered. Solvent production and glucose consumption rate at 24 h of fermentation along with the remaining glucose at 10 h were summarized in Table 3. The butanol production at 24 h increased in all co-culture experiments compared with the *Clostridium* monoculture (Table 3). Moreover, ABE production increased due to ethanol production by *S. cerevisiae*. In fact, the inoculation of the yeast simultaneously to *C. acetobutylicum* (0 h) led to mainly ethanol synthesis (ethanol/butanol ratio: 3.4 ± 0.1). The earlier presence of the yeast (0 h) caused a very early competition from the available sugar in the fermentation media between the two species, adversely impacting on butanol production. By postponing 5 h its inoculation the ethanol/butanol ratio decreased drastically (ethanol/butanol ratio: 1.1 ± 0.1), indicating that the conversion of glucose to acids by *Clostridium* was not hindered by *S. cerevisiae* growth. Further delay on the yeast inoculation (10 h) did not improve butanol proportion (ethanol/butanol ratio: 1.2 ± 0.2) while decreasing the overall glucose consumption rate. Although yeast inoculation time at 0 h gave better results in terms of ABE production, it was decided to delay 5 h the inoculation time of the yeast from the bacteria inoculation to promote butanol production over ethanol.

3.2.2 Effect of pH regulation on the co-culture

Batch reactors without pH control and by controlling $pH_{min} > 4.8$ were performed for co-culture fermentations using *C. acetobutylicum* and *S. cerevisiae*. The inoculation of *S. cerevisiae* was performed at 5 h after the beginning of the fermentation as indicated previously. The composition of the synthetic media (glucose: 35, xylose: 15 g L^{-1}; run 4) was equal to the concentration of the major sugars on the alkali-pretreated RS hydrolysate (Table 2), having a slightly lower content in glucose than the synthetic mixture used in previous experiments with the solely *Clostridium* strain (glucose: 45, xylose: 15 g L^{-1}). The co-culture fermentation patterns are depicted in Fig. 3. Regarding the uncontrolled pH experiment (Fig. 3a), it was observed the same acid crash phenomenon as without the use of *S. cerevisiae* (Fig. 1e); the pH decreased rapidly to 4.0, so glucose was not completely exhausted. However, a higher ABE production was achieved (9.3 ± 0.2 g L^{-1} g L^{-1} versus 7.7 g L^{-1}), indicating the beneficial effect by the presence of the yeast to improve the overall glucose uptake (from 66 to 83 ± 5%), even if an early acid crash occurred. Glucose consumption rate was also higher (1.65 ± 0.04 g L^{-1} h^{-1}) compared to the monoculture (1.35 g L^{-1} h^{-1}). The increment in solvents was mainly due to the increase in ethanol production by the alcoholic fermentation of *S. cerevisiae* (from 0.3 to 3.4 g L^{-1}). Butanol production versus total available glucose was only slightly higher in presence of the yeast than in its absence, as 4.0 ± 0.6 g L^{-1} of butanol with 35 g L^{-1} of glucose for the co-culture was obtained compared with 4.9 g L^{-1} of butanol with 45 g L^{-1} for the monoculture. In any case, these results showed that independently of the presence of the yeast, pH

Table 3 Effect of inoculation time of *S. cerevisiae* on solvent production (at 24 h) for the co-culture system

Experiment	Yeast Inoculation time (h)	Butanol (g L^{-1})	Ethanol (g L^{-1})	ABE (g L^{-1})	Ethanol/butanol ratio	Glucose $_{10\,h}$ (g L^{-1})	$-q_{glucose}$ (g L^{-1} h^{-1})
C. acetobutylicum monoculture	N/A	1.6 ± 0.3	0.2 ± 0.1	2.9 ± 0.5	0.1 ± 0.0	14.9 ± 0.2	0.72 ± 0.01
S. cerevisiae monoculture	N/A	0.0 ± 0.0	16.5 ± 0.4	16.5 ± 0.4	N/A	7.5 ± 2.4	1.17 ± 0.02
Coculture	0	2.0 ± 0.3	6.8 ± 1.1	10.0 ± 1.6	3.4 ± 0.1	6.6 ± 3.2	1.12 ± 0.00
Coculture	5	2.0 ± 0.2	2.3 ± 0.0	5.3 ± 0.3	1.1 ± 0.1	12.2 ± 1.1	0.96 ± 0.02
Coculture	10	1.8 ± 0.1	2.1 ± 0.3	4.9 ± 0.2	1.2 ± 0.2	13.4 ± 0.2	0.91 ± 0.02

Remaining glucose at 10 h is depicted. The ethanol/butanol ratio (*E*/*B* ratio) and the glucose consumption rate were calculated at 24 h

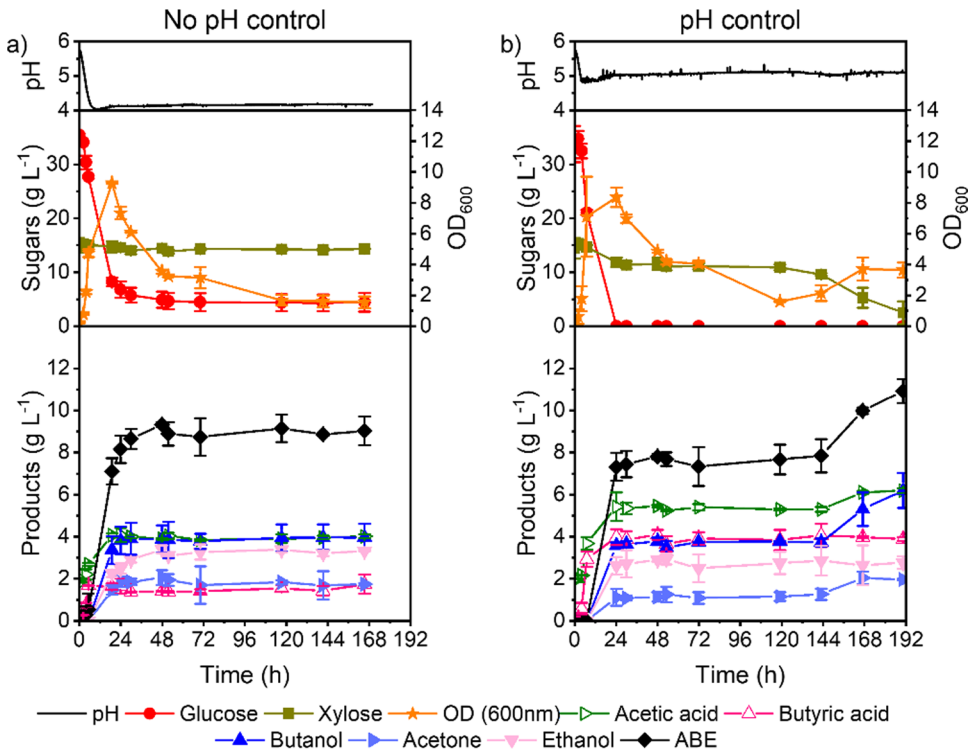

Fig. 3 Co-culture fermentation profiles by *C. acetobutylicum* DSM 792 and *S. cerevisiae* EYS4 with synthetic media (glucose: 35; xylose: 15 g L^{-1}). **a** no pH control and **b** pH control

regulation is required for a properly development of the *C. acetobutylicum* metabolism.

The profiles of sugar consumption and acid and solvent production during the co-culture fermentation under pH control are shown in Fig. 3b. The total solvent production was 10.9 ± 0.6 g L^{-1}, from which butanol was 6.2 ± 0.8 g L^{-1}. Unsuccessfully, ABE production versus total available sugar was equal to that observed with the solely *Clostridium* assay. Nevertheless, ABE composition slightly shifted to more ethanol ratio due to the competition of both species for the glucose. The reduction on butanol production considering the potential production if all sugars were metabolized by the *Clostridium* strain was of 1.2 g L^{-1}. Qi et al. [38] tested several combinations of elapsing times between inoculation of *C. acetobutylicum* and *S. cerevisiae* by using a mixture of 25:25 glucose:xylose. As we also observed, test butanol concentration was not improved in any of the co-culture assays comparing with the mono-*Clostridium* test. The best results in terms of ABE production obtained by these authors corresponded to the initial inoculation of *S. cerevisiae* followed by inoculation 24 h after *C. acetobutylicum.* By using this strategy, these authors slightly improved ABE production from 18.45 g L^{-1} (solely *C. acetobutylicum*) to 19.62 g L^{-1}. However, a drastic reduction in butanol production (5.29 vs 11.22 g L^{-1}) was given due to the faster metabolic rate of the yeast. In terms of butanol production, the delay in the yeast inoculation seems a better strategy.

Although the presence of the yeast did not reduce the adaptation time of the *Clostridium* strain to start xylose consumption, it enhanced the xylose consumption up to $85 \pm 13\%$. Positively, it was accompanied by a simultaneously increase of butanol up to 2.4 ± 0.8 g L^{-1} and with some acetone production (0.8 ± 0.2 g L^{-1}). The beneficial effect on the conversion of xylose to butanol by *C. acetobutylicum* would be linked to the ability of *S. cerevisiae* to secrete amino acids which could be uptake by the *Clostridium* species. In this regard, Wu et al. [26] monitored the secreted amino acids (aspartic, aliphatic, and aromatic acid family and arginine) by *S. cerevisiae* in a co-culture system with *C. beijerinckii*, observing, for example, an increment of 150% for phenylalanine concentration over the *Clostridium* monoculture. The assimilation of amino acids by *Clostridium* species would increase monosaccharide intracellular transportation and butanol tolerance [26, 27]. This evidence opened to test the co-culture system in more realistic conditions.

The profiles of sugar utilization and product formation during the co-culture fermentation of alkali-pretreated RS hydrolysate (run 5) are shown in Fig. 4. As in the previous experiments occurred, acid crash impeded an adequate development of the solventogenesis under lack of pH control (Fig. 4a), corroborating the importance of controlling pH in such system. By controlling the pH, it was achieved the highest solvent production among the experiments with the same initial sugar levels (ABE: 13.1 ± 0.1, from which butanol: 7.0 ± 0.4, acetone: 3.4 ± 0.2, and ethanol: 2.7 ± 0.7 g L^{-1}). Successfully, the *S. cerevisiae* and *C. acetobutylicum* co-culture was able to increase not only ABE, but also slightly the butanol production. The enhancement

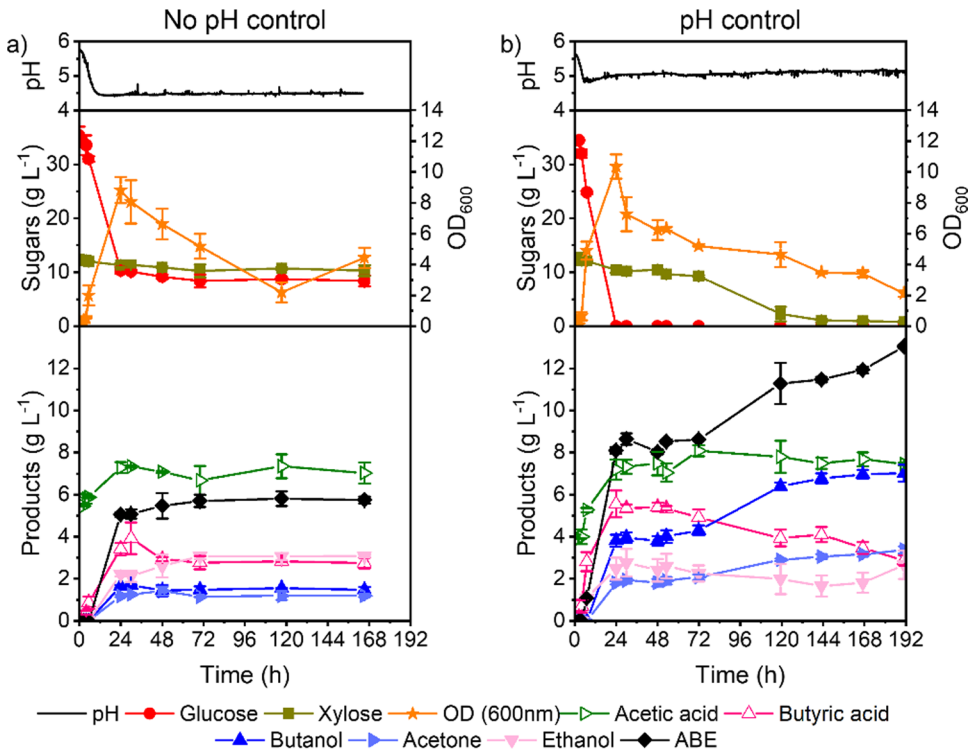

Fig. 4 Co-culture fermentation profiles by *C. acetobutylicum* DSM 792 and *S. cerevisiae* EYS4 with RS hydrolysate. **a** no pH control and **b** pH control

of the ethanol production (~3.0-fold) in this co-culture fermentation is of importance for the better exploitation of the RS hydrolysate. The use of alkali RS hydrolysate in the co-culture alleviated in some extent the lag phase of the xylose metabolism, as the consumption started at ~72 h instead of ~120 h. At the end of the fermentation, the maximum xylose uptake (94 ± 1%) was detected, which was ~2.0-fold over the RS hydrolysate monoculture. At the same time, ABE production increased suddenly up to 4.4 ± 0.1 (butanol: 2.7 ± 0.2, acetone: 1.3 ± 0.0, ethanol: 0.4 ± 0.3) g L^{-1}. The better results in comparison with the synthetic media can be associated to the complex chemical composition of the RS hydrolysate. In this sense, Jin et al. [39] observed a faster sugar consumption when fermenting apple pomace residue compared with using a sugar solution; furthermore, Moradi et al. [40] produced more butanol when fermenting with alkali-pretreated RS hydrolysate over a pure sugar medium. Literature regarding the utilization of co-cultures with *Clostridium* and *S. cerevisiae* is still scarce and most of them used starchy substrates (Table 4). In our case, the combination of an adequate pH regulation and the co-culture system with RS hydrolysate substantially boosted the consumption of xylose, which allowed the better use of the substrate (94% over the 47% of the monoculture). This led to an increase on ABE production of about 38% (Table 4). The ABE yield remained similar due to simultaneous increment in ABE production and sugar consumption. Maximum ABE productivity remained constant over the *Clostridium* monoculture as it was associated to glucose. The use of co-cultures with starchy substrates up to 150 g L^{-1} also exhibited an increment in total ABE production in a range of 37 to 116% over its monocultures (Table 4) [27, 29, 41]. These easier assimilable residues composed mainly by glucose led to increments in either the ABE yield [29] or the productivities [27, 41] (Table 4). In the sense of lignocellulosic substrates, it seems that the *Clostridium* species has a significant impact. Using *C. beijerinckii* the butanol production increased from 4.22 to 10.62 g L^{-1} by implementing the co-culture system with *S. cerevisiae* [42], which was not evident with *C. acetobutylicum*. Our results support that the co-culture strategy with *Clostridium* species and *S. cerevisiae* can extend ABE concentrations not only with starchy substrates, but also with lignocellulosic wastes. Furthermore, this co-culture strategy enhances the exploitation of the substrate.

4 Conclusions

The main aim of this work was to evaluate the integration of pH regulation and a co-culture system of *C. acetobutylicum* and *S. cerevisiae* with the target to improve xylose consumption and subsequent solvent concentrations from RS hydrolysate**.** The modulation of pH by using ammonium acetate as buffer and active minimum pH control at 4.8 was successfully applied to RS hydrolysate. The use of the co-culture of *C. acetobutylicum* and *S. cerevisiae* led to an increase of 1.4-fold of the total solvent concentration, mainly due to ethanol production. Moreover, *S. cerevisiae* promoted the

Table 4 Comparison of production performance from *Clostridium and S. cerevisiae* co-cultures

Substrate/sugar concentration (g L^{-1})	Microorganism	Production (g L^{-1})			Yield (g g^{-1})		Maximum productivity (g L^{-1} h^{-1})		pH control	References
		Butanol	ABE	ABE increment (%)	ABE	ABE increment (%)	ABE	ABE increment (%)		
Rice straw/58	*C. acetobutylicum* DSM 792	6.50	9.50	38	0.24	9	0.35	0	Yes	This study
Rice straw/58	*C. acetobutylicum* DSM 792/*S. cerevisiae* EYS4	7.00	13.10		0.28		0.35		Yes	This study
Corn flour/150	*C. acetobutylicum* ATCC 824	11.63	18.77	37	0.37	−2	0.29	66	No	[27]
Corn flour/150	*C. acetobutylicum* ATCC 824/*S. cerevisiae*	11.91	25.69		0.36		0.48		Yes	[27]
Corn flour/150	*C. acetobutylicum* ATCC 824	7.67	12.70	47			0.25	31	No	[41]
Corn flour/150	*C. acetobutylicum* ATCC 824/*S. cerevisiae*	9.53	18.61				0.33		No	[41]
Cassava chip flour/150	*C. acetobutylicum* CH02	13.36	19.70	116	0.32	44			No	[29]
Cassava chip flour/150	*C. acetobutylicum* CH02/*S. cerevisiae*	11.11	42.56		0.46				No	[29]
Rice straw/~45	*C. beijerinckii F-6*	4.22							No	[42]
Rice straw/~45	*C. beijerinckii F-6*/*S. cerevisiae*	10.62							No	[42]

xylose uptake by *C. acetobutylicum*, probably associated due to the amino acids excreted by the yeast, thus enhancing the overall exploitation of the RS. The better exploitation of the secondary monosaccharaide of hydrolysates, xylose, in the co-culture was correlated to butanol production by *Clostridium* species. This co-culture fermentation strategy can be used to increment butanol and ethanol co-production and sugar consumption from lignocellulosic wastes.

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Declarations

Conflict of interest The authors declare no competing interests.

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